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CONTENTS

THE CALCULATION OF BIOLOGICAL ASSAY RESULTS BY GRAPHIC METHODS. THE ALL-OR-NONE TYPE OF RESPONSE[1]

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The all-or-none type of response is frequently encountered in biological assay work and in the evaluation of certain pharmacological properties of new drugs. Trevan (1), Gaddum (2), Bliss (3) and others have pointed out that the successful treatment of such data must not only express potency by some particular dose such as the LD_{50}, but also, because of uncontrollable animal variations, must give some idea of the precision of this figure. The following three values are particularly useful: (1) the LD_{50}, which depends upon the over-all susceptibility of the animals used, (2) the slope, which depends upon the uniformity of response, and (3) the error associated with the potency figure, which depends upon the standard error and the slope. A particularly comprehensive treatment fulfilling these requirements has been given by Bliss (4, 5, 6). For a discussion of the principles underlying the various formulas, these references may be consulted.

The purpose of the present paper is to describe the construction and use of a series of scales and nomographs by means of which the essential computations described by Bliss may easily and rapidly be done without the aid of a calculating machine. Other less extensive graphic treatments have been described by Litchfield and Fertig (7) and by Miller (8).

THE PROBIT. In dealing with the all-or-none type of response, experiments may be so designed that a certain proportion of a group of animals receiving a given dose will be found to give a positive reaction. This proportion may be expressed as a percentage. The percentage in turn may be expressed as a probit, which is a function of the standard deviation. Tables of probits giving the values corresponding to various percentages are given by Bliss (6). For example, a certain dose of digitalis tincture may kill 4 out of 10 frogs injected. The percentage would be 40 and the corresponding probit is 4.75. Many dose-response curves become straight lines when the probit is plotted against the log dose and the calculations may be greatly simplified when the data are expressed in this fashion.

THE BASIC GRAPH. The first step in carrying out the computations is to plot the probit against the log-dose. Several years of experience have shown that a basic graph of the following dimensions is suitable for most types of data.

Each probit unit is made 5 cm. long and each logarithm unit (*i.e.*, from 0.000 to 1.000) is made 50 cm. long. The probit scale can then be subdivided into hundredths and the log scale into thousandths As a further convenience, the logarithms are also marked off (as on a slide rule) with the corresponding numbers and the probits with the corresponding percentages. (See figure 1.) These double scales do away with the necessity of using log tables or probit tables. In order to protect this graph it may be mounted on a light board and covered with inexpensive transparent plastic sheeting. This sheeting also affords a means of using the basic chart repeatedly since the various points may be plotted on the surface of the plastic with a wax marking pencil and after the results
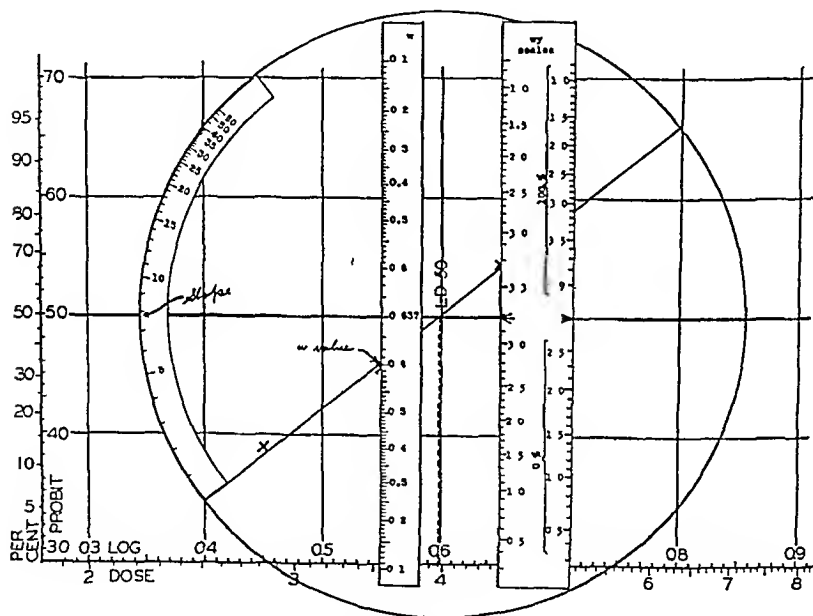
Fig 1. The Basic Graph
The regression line and the use of the scales for slope, for *w* and for *wy* are shown

have been calculated, the marks can easily be removed. Along the bottom edge of the chart, parallel to the abscissa, a small piece of molding may be fastened in order to enable the various scales (to be described later on) to be placed in position rapidly and easily.

THE REGRESSION LINE AND THE LD$_{50}$ VALUE. After plotting the points, the dose-response curve or regression line is established by fitting the best straight line by inspection. The line may then be drawn on the chart or established by the convenient device illustrated in figure 1. This is constructed by inscribing a straight line along the diameter of a circular, transparent, plastic disk, which

is just large enough to have its center always fall on the probit-5 line when the bottom edge rests against the molding. The point of intersection of the regression line with the probit-5 line locates the LD$_{50}$ which may be read directly below on the dose scale. See figures 1 and 4.

THE SLOPE. The slope may be read directly with the aid of the protractor described in a previous communication (9) or by means of the modification illustrated in figure 1. The modified scale is constructed in the same manner as the protractor, save that the readings run clockwise. It is mounted face upward with the aid of clear lacquer on the underside of the plastic disk described in the preceding paragraph. The zero of the slope is located at the regression line. The slope is read directly from the intersection of the slope scale with the probit-5 line. The advantage of the modified scale is that the fitting of the regression line automatically sets the slope scale.

THE WEIGHTING COEFFICIENT SCALE. The weighting coefficients, w, tabulated by Bliss (6), take into consideration the fact that responses are of greatest significance at probit 5 and that they progressively decrease in weight as they approach 0 or 100 per cent. The w values may be rapidly obtained with the aid of a special scale constructed from table 2, which gives the probit corresponding to a given set of w values, and which is a transformation of Bliss' table of weighting coefficients. To construct the scale, place a paper strip along the ordinate axis of the basic graph and mark off the w values at their proper probits. For example, the weight $w = 0.637$ is marked off at probit 5.00; the weight $w = 0.4$ is marked off at probits 3.89 and 6.11, since $w = 0.4$ at both of these probits, etc. (See figure 1.)

To use the weighting scale, place it vertically on the basic graph parallel to the ordinate at the given dose (so that the point at which $w = 0.637$ falls on the probit 5 line), and read the w value directly from the scale at the point where it is intersected by the regression line. (See figure 1.) Multiply the w value by the number of animals receiving the dose in order to get the term wn. The wn value is obtained for each dose and all of the wn values are then added together to obtain the sum $S(wn)$. Approximate weights for 0 and 100 per cent responses may also be obtained in this way, although for greater exactness a special scale may be constructed from Bliss' tables (6).

THE STANDARD ERROR OF THE LOG LD$_{50}$. The following formula for the standard error of the log LD$_{50}$ may be greatly simplified if the experiment is well planned:

$$s = \frac{1}{b} \sqrt{\frac{(5 - \bar{y})^2}{b\{S(wnxy) - \bar{y}S(wnx)\}} + \frac{1}{S(wn)}}$$

Here b represents the slope, S the summation symbol, w the weighting coefficient based upon the expected response in (probits) to a given dose, n the number of animals receiving the dose, x the log dose, y the corresponding probit and $\bar{y}$ the mean of all the probits. In a well designed experiment the entire fraction

$$\frac{(5 - \bar{y})^2}{b\{S(wnxy) - \bar{y}S(snx)\}}$$

may be safely omitted, since under these conditions the percentage responses will be balanced above and below 50 per cent and the average of all the probits will approach 5; consequently the value of the fraction will approach zero. The formula then reduces to the comparatively simple form below which can readily be calculated with the aid of a weighting scale and a nomograph:

$$s = \frac{1}{b\sqrt{S(wn)}}$$

THE NOMOGRAPH FOR THE STANDARD ERROR OF THE LD_{50}. After having obtained the term $S(wn)$, the standard error formula $s = 1/\{b\sqrt{S(wn)}\}$ may be
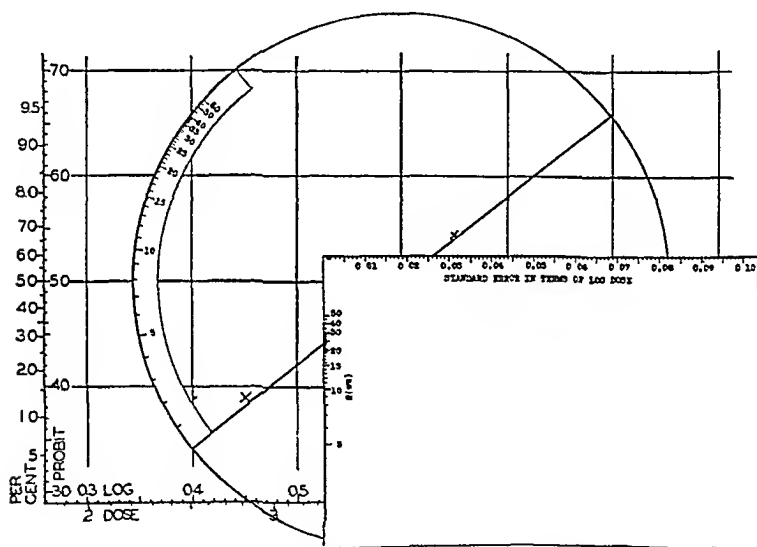
FIG. 2. THE COMPUTATION OF THE STANDARD ERROR

rapidly solved by the nomograph shown in figure 2. To construct this nomograph, first lay off vertically a preliminary probit scale with equally spaced divisions, zero being at the upper edge of the paper. Mark off permanently the consecutive whole number values of $S(wn)$ at the corresponding probit values as obtained from table 2, and then remove the preliminary scale. The resulting scale which no longer has equally spaced divisions automatically converts $S(wn)$ into $1/\sqrt{S(wn)}$ giving the values in terms of probits. The probit values are next changed into useful log dose units by dividing by the slope. This is accomplished by means of the horizontal scale which is divided into log units.

In order to make the nomograph more accurate, the divisions of the preliminary probit scale may be made several times larger (1.probit = 20 cm.)

than that of the basic chart. However, the horizontal log dose scale must also be made correspondingly larger so that the ratio between the length of probit units and log dose units is the same as that of the basic chart.

To use this nomograph, place the horizontal scale parallel to the log-dose scale of the basic chart and allow the vertical scale to intersect the regression line at the $S(wn)$ value obtained previously. (See figure 2.) It will be found that the regression line will also intersect the log-dose scale and at this point the standard error, s, may be read directly in terms of logarithms. For practical purposes this figure may be converted into limits of error, by which term is meant the region within which the LD_{50} may be reasonably expected to fall, *i.e.* about 21 out of 22 times. The following expressions give the limits as approximate percentages to be applied to the LD_{50} value.

$$\text{Upper limit} = 100 \text{ antilog } 2s$$
$$\text{Lower limit} = 100 \, (1/\text{antilog } 2s)$$

The simple nomograph of figure 3 converts the standard error into upper and lower limits directly. It was constructed by plotting whole numbers for the upper limits and reciprocals of numbers for the lower limits opposite one-half of their mantissa on the middle logarithmic scale.

FIG. 3. A NOMOGRAPH FOR TRANSFORMING THE STANDARD ERROR INTO LIMITS OF ERROR

BIOLOGICAL ASSAYS. In biological assay work the potency of the substance being tested is customarily determined in terms of a standard. This involves the use of two regression lines, one for the standard and one for the material being tested, as well as values for the slope, b; the standard error, s; the weighted mean response $\bar{y}$, and the weighted mean log dose, $\bar{x}$. The methods for obtaining the slope and the standard error have been described.

THE WEIGHTED MEAN RESPONSE OR $\bar{y}$. The weighted mean response, $\bar{y}$, may be readily computed with the aid of a movable wy scale similar to the u scale. To construct the wy scale, place a paper strip along the probit axis of the basic graph and mark off the wy values of table 2 at their proper probits.

Like the w scale, the wy scale is used by placing it vertically at the various given doses and so that the value for $wy = 3.17$ falls on the 50 per cent line. See figure 1. The wy value for the dose in question is then read from the point of intersection of the regression line with the wy scale. It is multiplied by n, the number of animals receiving the dose and all of the wny values are summed up to obtain the sum of the weighted responses, $S(wny)$. From the following formula $\bar{y}$, in probits, is easily obtained.

$$\bar{y} = \frac{S(wny)}{S(wn)}$$

It occasionally happens that a zero or a one hundred per cent response is obtained. This case is discussed by Bliss (6) who gives a special set of probits to be used with this type of data.

The wy scales for zero and one hundred per cent responses are constructed from the data given in table 2 in the same manner as the wy scale described above.

In actual use, when a zero per cent response is obtained, the wy scale for 0% is erected at the dose given and, if it intersects the regression line, the appropriate wy value is read from the point of intersection. The wy value obtained is multiplied by n and treated as above. When the response is one hundred per cent, the 100% wy scale is used.
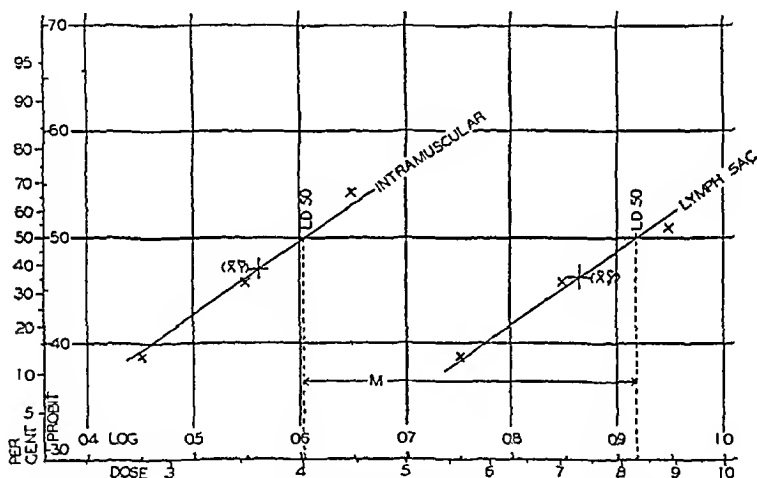
FIG. 4. THE COMPUTATION OF RELATIVE POTENCY FROM TWO DOSE-RESPONSE CURVES

THE WEIGHTED MEAN LOG DOSE OR $\bar{x}$. The dose corresponding to the point $\bar{y}$ on the regression line will be $\bar{x}$. It may be read directly from the graph. (See figure 4.)

The point whose coördinates are $(\bar{x}, \bar{y})$ is the most important point on the dose-response curve, since all of the data for the drug contribute toward fixing its position.

THE COMMON SLOPE OR b_c. It is commonly assumed that the behavior of the test animal will be the same toward both the standard and the unknown. Therefore, any difference in slope is to be ascribed to animal variation and a common slope, b_c, may be calculated by averaging the two slope values. If, however, one curve happens to be much better established than the other, a better estimation of b_c may be obtained by combining the weighted b values as follows:

$$b_c = \frac{b_s\{S(wn)_s\} + b_u\{S(wn)_u\}}{S(wn)_s + S(wn)_u}$$

The subscripts s and u refer to the standard and the unknown respectively.

DETERMINATION OF M AND CALCULATION OF THE RELATIVE POTENCY. Each regression line is then so adjusted that it passes through its respective $(\bar{x}, \bar{y})$ point and so that the slope as read from the slope scale is equal to b_c. The horizontal distance between the two, now parallel, regression lines is equal to M, the logarithm of the ratio of the potencies. Reading along the probit 5 line, $M = \log LD_{50s} - \log LD_{50u}$. The LD_{50} values may be read directly from the basic chart. The antilog of M, multiplied by 100, gives the potency of the unknown as a percentage of the standard.

THE LIMITS OF ERROR OF THE ASSAY. The usual formula for the standard error of the difference between two means will give a good approximation of the standard error of M, unless the difference between the two mean responses, $\bar{y}_s - \bar{y}_u$, is great, in which case the approximation will be too low.

$$s_M = \sqrt{s_s^2 + s_u^2}$$

In this formula for the standard error of M, the values s_s and s_u are the respective standard errors of LD_{50} for the standard and the unknown respectively. s_M which is a logarithm may be transformed into limits of error as shown below.

Upper limit = 100 (antilog $2s_M$)
Lower limit = 100 (1/antilog $2s_M$)

All of these calculations may be performed with the nomograph of figure 5. To construct the nomograph, square consecutive values of the standard errors, *i.e.* 0.001, 0.002, 0.003 ... 0.1. The squares are then located along arithmetically spaced divisions (for example, 1 cm. = 0.000001) on the two outside lines of a set of three equidistant parallel lines. The number itself is then written opposite its square. Table 2 gives the lower and upper limits of error and these figures are laid off on the right and left hand sides of the middle line. For example, an upper limit figure of 120 falls on a line joining the two standard error figures of 0.028 on the two outside lines.

To use this nomograph, join the observed s_s and s_u values with a straight edge and read off the limits of error directly. This nomograph squares each standard error, adds the squares, takes the square root of the sum, multiplies by 2, takes the antilogarithm, multiplies it by 100 for the upper limits and finds its reciprocal and multiplies by 100 for the lower limits.

EXAMPLE OF CALCULATIONS. The detailed calculations presented in a paper by Miller, Bliss and Braun (10) on the use of the frog for the assay of digitalis present a good means of checking the various steps of the graphic calculations. Graded doses of a digitalis preparation were injected into groups of 15 frogs by two different routes and the number of fatalities was determined. In table 1 all of the graphic calculations are recorded in bold faced type and the corresponding machine calculated figures, in parentheses, are placed to the right or below

for the sake of comparison. In this example the agreement is very good because the experimental data closely fit the two regression lines. (See table 1.) The limits of error are not given as such by Miller, Bliss and Braun, but may be easily calculated by substituting 0.0423, their value for the standard error of M, in the limits of error formulas given previously.
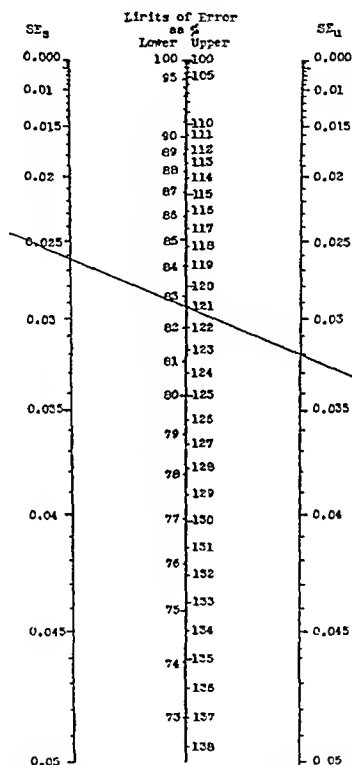
FIG 5 A NOMOGRAPH FOR COMPUTING THE LIMITS OF ERROR OF AN ASSAY

The data of table 1 may be selected as an example of a situation which is frequently encountered during the investigation of new drugs. Here toxicity values and other pharmacological properties must be expressed on an absolute basis because of the lack of a suitable standard. In such cases it is usually sufficient to fix the dose-response curve by giving the LD_{50}, or an analogous figure, and the slope. The precision is indicated by giving the limits of error.

The "Intramuscular" data are plotted as in figure 1 It is unnecessary to consult tables of probits or logarithms since the basic graph automatically converts percentage into probits and doses into their logarithms The dose-response curve, or regression line, is fitted by inspection, and the LD_{50}, as read

from the point of its intersection with the probit 5 line, is seen to be 3.96. The intersection of the protractor scale with the probit 5 line shows the slope to be 7.7.

The limits of error of the assay are obtained by using the w scale and the standard error nomograph. In figure 1 it will be seen from the intersection of the w scale and the regression line that the w value for dose 3.55 is 0.6 which, when multiplied by 15, the total number of animals receiving this dose, gives a weight of 9.0. The weights for the other two points are found in similar fashion and added to obtain $S(wn)$, i.e. $5.6 + 9.0 + 9.0 = 23.6 = S(wn)$. The right-angled, standard error, nomograph is placed as in figure 2, so that its vertical

TABLE 1

EXPERIMENTAL				CALCULATIONS		
Injection Route	Dose	Response	Killed	wn		wny
	cc./kgm.		%			
(Unknown) Intramuscular	2.82	2/15	13.3	5.6	22.0	$LD_{50} = 4.01$
	3.55	5/15	33.3	9.0	41.3	$\log LD_{50} = 0.603$
	4.47	10/15	66.7	9.0	48.4	$b = 7.7$ (7.8)
				23.6	111.7	$\bar{y} = 4.73$ (4.73)
				(24.0)	(113.2)	$\bar{x} = 0.559$ (0.563)
						$s_u = 0.0261$
(Standard) Lymph Sac	5.62	2/15	13.3	6.0	23.3	$LD_{50} = 8.34$
	7.08	5/15	33.3	8.7	39.0	$\log LD_{50} = 0.921$
	8.91	8/15	53.3	9.5	48.3	$b = 6.2$ (5.9)
				24.2	110.6	$\bar{y} = 4.57$ (4.60)
				(24.5)	(112.7)	$\bar{x} = 0.860$ (0.863)
						$s_x = 0.0320$

$b_c = \dfrac{6.2 + 7.7}{2} = 7.0$ (6.8)

$M = 0.921 - 0.603 = 0.318$ (0.319)

Potency = 100 antilog M = 208% (209%)

Limits of error = 83–121% (82–121%)

scale is parallel to the probit axis of the basic graph and so that 23.6, the value found above for $S(wn)$, falls on the point of intersection of the regression line with the $S(wn)$ scale. The standard error, s, expressed in logarithms, is then read directly from the intersection of the regression line with the horizontal scale. In figure 2 it will be seen to be 0.0261. This is transformed into limits of error with the aid of the scale of figure 3 or by substituting in the simple formulas previously given, i.e.:

$$\text{Upper limit} = 100\{\text{antilog}(2 \times 0.0261)\} = 113\%$$
$$\text{Lower limit} = 100/\text{antilog}(2 \times 0.261) = 89\%$$

It will be observed that, on the basis of chance alone, the LD_{50} may be expected to fall anywhere within the limits of 89 to 113 per cent of the value obtained.

TABLE 2

WEIGHTING SCALE

Pro-bit	w	Pro-bit	w	Pro-bit
5.00	0.637	3.77	0.36	6.23
4.83	0.63	3.74	0.35	6.26
4.73	0.62	3.71	0.34	6.29
4.66	0.61	3.68	0.33	6.32
4.60	0.60	3.65	0.32	6.35
4.55	0.59	3.63	0.31	6.38
4.50	0.58	3.60	0.30	6.41
4.45	0.57	3.57	0.29	6.44
4.41	0.56	3.54	0.28	6.47
4.37	0.55	3.50	0.27	6.50
4.33	0.54	3.47	0.26	6.53
4.30	0.53	3.44	0.25	6.56
4.26	0.52	3.41	0.24	6.59
4.23	0.51	3.37	0.23	6.63
4.19	0.50	3.34	0.22	6.66
4.16	0.49	3.31	0.21	6.69
4.13	0.48	3.27	0.20	6.73
4.10	0.47	3.24	0.19	6.76
4.06	0.46	3.20	0.18	6.80
4.03	0.45	3.16	0.17	6.84
4.00	0.44	3.12	0.16	6.88
3.98	0.43	3.09	0.15	6.92
3.95	0.42	3.04	0.14	6.96
3.92	0.41	3.00	0.13	7.01
3.89	0.40	2.95	0.12	7.05
3.86	0.39	2.90	0.11	7.10

WEIGHT X PROBIT SCALE

Pro-bit	wy	Pro-bit	wy
5.04	0.3	2.87	3.2
5.27	0.4	3.01	3.2
5.50	0.5	3.12	3.2
5.63	0.6	3.22	3.1
5.72	0.7	3.31	3.0
5.81	0.8	3.39	2.9
5.88	0.9	3.47	2.8
5.95	1.0	3.54	2.7
6.02	1.1	3.61	2.6
6.08	1.2	3.67	2.5
6.14	1.3	3.74	2.4
6.20	1.4	3.80	2.3
6.26	1.5	3.86	2.2
6.31	1.6	3.91	2.1
6.36	1.7	3.97	2.0
6.42	1.8	4.03	1.9
6.47	1.9	4.08	1.8
6.53	2.0	4.14	1.7
6.58	2.1	4.20	1.6
6.64	2.2	4.25	1.5
6.70	2.3	4.31	1.4
6.75	2.4	4.37	1.3
6.81	2.5	4.43	1.2
6.88	2.6	4.49	1.1
6.94	2.7	4.56	1.0
7.00	2.8	4.63	0.9

WEIGHT X PROBIT SCALES

0% response		100% response	
wy	Pro-bit	wy	Pro-bit
0.3	2.95	3.9	5.28
0.4	3.09	3.8	5.41
0.5	3.12	3.7	5.50
0.6	3.31	3.6	5.59
0.7	3.41	3.5	5.66
0.8	3.50	3.4	5.73
0.9	3.58	3.3	5.79
1.0	3.66	3.2	5.85
1.1	3.73	3.1	5.90
1.2	3.81	3.0	5.96
1.3	3.88	2.9	6.01
1.4	3.95	2.8	6.06
1.5	4.02	2.7	6.11
1.6	4.09	2.6	6.16
1.7	4.17	2.5	6.21
1.8	4.24	2.4	6.25
1.9	4.32	2.3	6.30
2.0	4.41	2.2	6.35
2.1	4.50	2.1	6.40
2.2	4.60	2.0	6.45
2.3	4.73	1.9	6.50
		1.8	6.55
		1.7	6.60
		1.6	6.64
		1.5	6.69
		1.4	6.74

STANDARD ERROR SCALE

$S(\bar{m})$	Pro-bit	$S(\bar{m})$	Pro-bit
5	0.447	30	0.183
6	0.408	31	0.180
7	0.378	32	0.177
8	0.354	33	0.174
9	0.333	34	0.172
10	0.317	35	0.169
11	0.302	36	0.167
12	0.280	37	0.164
13	0.277	38	0.162
14	0.267	39	0.160
15	0.258	40	0.158
16	0.250	41	0.156
17	0.243	42	0.154
18	0.236	43	0.153
19	0.229	44	0.151
20	0.224	45	0.149
21	0.218	46	0.147
22	0.213	47	0.146
23	0.209	48	0.144
24	0.204	49	0.143
25	0.200	50	0.141
26	0.196	55	0.135
27	0.193	60	0.129
28	0.189	65	0.124
29	0.186	70	0.120

LOWER LIMIT OF ERROR

Lower limit	Standard error	Lower limit	Standard error
100	0.0000	83	0.02863
99	0.0015	82	0.03053
98	0.0030	81	0.03241
97	0.0047	80	0.03426
96	0.0063	79	0.03622
95	0.0079	78	0.03815
94	0.0095	77	0.04017
93	0.0111	76	0.04217
92	0.0128	75	0.04413
91	0.0145	74	0.04620
90	0.0162	73	0.04831
89	0.0180	72	0.05045
88	0.0196	71	0.05254
87	0.0213	70	0.05481
86	0.0232	69	0.05695
85	0.0249	68	0.05926
84	0.0267	67	0.06154

UPPER LIMIT OF ERROR

Upper limit	Standard error	Upper limit	Standard error
100	0.0000	126	0.0355
101	0.0015	127	0.0367
102	0.0030	128	0.0379
103	0.0045	129	0.0391
104	0.0060	130	0.0403
105	0.0075	131	0.0415
106	0.0090	132	0.0426
107	0.0104	133	0.0438
108	0.0118	134	0.0449
109	0.0132	135	0.0461
110	0.0146	136	0.0472
111	0.0160	137	0.0483
112	0.0174	138	0.0495
113	0.0188	139	0.0506
114	0.0201	140	0.0517
115	0.0215	141	0.0528
116	0.0228	142	0.0538
117	0.0241	143	0.0549
118	0.0254	144	0.0560
119	0.0267	145	0.0571
120	0.0280	146	0.0581
121	0.0293	147	0.0592
122	0.0305	148	0.0602
123	0.0318	149	0.0612
124	0.0330	150	0.0623
125	0.0343		

3.83	0.38	6.17	2.85	0.10	7.15	2.9	4.70	0.8	7.08
3.80	0.37	6.20				3.0	4.79	0.7	7.16
						3.1	4.90		

1.3	6.80
1.2	6.86
1.1	6.92
1.0	6.98
0.9	7.05
0.8	7.12

The weighting scale. The probits corresponding to a given set of w values are given. The figures were obtained by graphic interpolation of Bliss' tables.

The weight by probit scale. Values of the products of various weights by their corresponding probits are listed opposite the corresponding probits. The figures were obtained by graphic interpolation of graphs relating wy to y. The 0% and 100% values were obtained in like manner.

The standard error scale. The probits corresponding to various values of $1/\sqrt{S(wn)}$ are listed opposite their respective $S(wn)$ values. The figures were calculated by substituting values of $S(wn)$ in the expression $1/\sqrt{S(wn)}$. The figures were calculated

The lower limit of error. The lower limits of error are listed opposite the corresponding standard errors. For purposes of calculation the case in which $s_e = s_u$ may be used. with the aid of the expression, lower limit $= 100/\text{antilog} \left(2\sqrt{s_e^2 + s^2}\right)$. The equation may then be simplified to lower limit $= 100/\text{antilog} \left(2 \times s \times \sqrt{2}\right)$.

The upper limit of error. The upper limits of error are listed opposite the corresponding standard errors. Calculations were made in a manner analogous to that used for the lower limits of error. The equation used was

upper limits $= 100 \text{ antilog} \left(2\sqrt{s_e + s_w}\right)$

The data of the table may also be used as an illustration of the type of bioassay in which the potency of a substance is determined in terms of a suitable standard. In this case the intramuscular data will serve as the unknown. The data are plotted as before and $S(wn)$ and the slope are recorded. Next, with the aid of the wy scale, the weighted response is found for each dose. In figure 1, it will be seen that when the wy scale is erected at the dose of 4.47, the intersection of the regression line with the scale gives a figure of 3.23 which, when multiplied by 15, the number of animals receiving the dose, gives a weighted probit of 48.4. The weighted probits for the other two points are found in similar fashion and added to obtain $S(wny)$, $i.e.$, $22.0 + 41.3 + 48.4 = 111.7$. When $S(wny)$ is divided by $S(wn)$, the mean weighted response, $\bar{y}$, is obtained or $\bar{y} = 111.7 \div 23.6 = 4.73$. The average log dose, $\bar{x}$, corresponding to the average weighted response as read directly from the graph is found to be 0.559. In the same manner $\bar{y}$, $\bar{x}$, and the slope are found to be 4.57, 0.860 and 6.2 respectively, for the standard or lymph sac injection data. The two slope values when averaged together give 7.0 for b_c.

Next the regression line is adjusted so that its slope is equal to 7.0 and so that it passes through the point, $(\bar{x}, \bar{y})$, whose coördinates are 0.860 and 4.57, and the log LD_{50} for the lymph sac data is found to be 0.921.[2] In similar fashion the log LD_{50} for the intramuscular data is seen to be 0.603. The potency of the "unknown" (intramuscular data) in terms of the standard (lymph sac data) is the antilog of the difference between these two figures. As table 1 shows, the potency is 208%. See figure 4.

The limits of error of this value were obtained by setting the regression line so that its slope was 7.0, the value for b_c, and using the standard error nomograph as previously described. The two standard error figures were then found to be $s_s = 0.0261$ and $s_u = 0.0320$. These two figures were applied to the limits of error nomograph as in figure 5, and the limits of error were read directly as 83–121%.

<div align="center">SUMMARY</div>

1. The LD_{50}, slope and error of dose-response curves for the all-or-none type of data may be rapidly calculated with the aid of a series of scales and nomographs.

2. The potency and its associated error for assays involving data of the all-or-none type may also be calculated with these scales.

3. The construction of the scales and nomographs is described and their use is illustrated by an example.[3]

[2] It will be observed that the LD_{50} corresponding to the antilog of 0.921 when obtained in this fashion may be slightly different than when the intramuscular data is considered alone, since the regression line passes through the point $\overline{xy}$ in each case, while the common slope, 7.0 is somewhat different from the slope of 7.7 for the intramuscular data when used alone.

[3] A limited number of full size sets of these scales and nomographs are available for distribution.

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ANTITHYROID STUDIES

II. The Goitrogenic Activity of Some Imidazoles and Benzimidazoles

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In the previous publication (1) it was shown that benzimidazole-2-thiol is approximately eight times more active as an antithyroid agent in the rat than phenylthiourea, ring closure apparently inducing the enhanced effect.
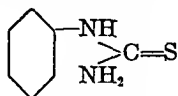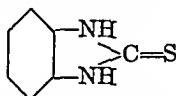
Phenylthiourea	Benzimidazole-2-thiol (keto form)
(14)	(116)

Furthermore the data demonstrated that benzimidazole-2-thiol having an activity of -116 was somewhat more potent than thiouracil which was assigned an activity of 100. It was desirable, therefore, to investigate derivatives and some isologs of benzimidazole-2-thiol, in order to establish the effect of substitution upon its activity. For this purpose several new benzimidazole-2-thiols were synthesized and tested for antithyroid activity by the method previously described (1). A number of 2-amino-benzimidazoles were included in this study to establish the effect of removing the mercapto group from the molecule.

The above relationship between phenylthiourea and benzimidazole-2-thiol suggested that similar relationships in the aliphatic substituted thioureas should also be investigated. For example, cyclization of ethyl thiourea would lead to 2-imidazoline-2-thiol:
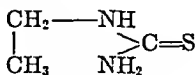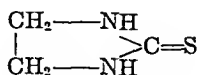
Ethylthiourea	2-Imidazoline-2-thiol (keto form)
(35)	(63)

Therefore 2-imidazoline-2-thiol was tested to determine whether cyclization in this case also led to increased activity. The experimental results show that such was indeed the case.

CHEMISTRY. The benzimidazole-2-thiols described in table 1 were prepared by two methods: (A) by fusing the appropriate *o*-phenylene diamine, or its dihydrochloride, and thiourea until evolution of gas ceased (2) and (B) by refluxing the *o*-phenylene diamine with carbon disulfide in alcoholic sodium or potassium hydroxide solution for several hours. This procedure is essentially that of Fischer (3) except that alkali was used in the reaction.

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TABLE 1

Benzimidazole-2-thiols
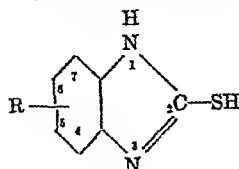
R	METHOD	% YIELD	M.P.*	ANALYSIS % N†	
				Calc.	Found
			°C.		
H‡	A	67.4	309–310	18.65	18.74
	B	84.0	312–313		18.58
1-Methyl	A	15.2	200.0–200.5	17.06	17.39
					17.41
1-n-Butyl	A	7.2	100–101	13.71	13.69
5(6)-Methyl	A	20.0	>295 (dec.)§	17.06	17.24
					17.10
5(6)-Methoxy	A	25.2	261–263	15.54	15.31
	B	67.6	261		15.33
5(6)-Ethoxy	A	22.0	251–252	14.42	14.46
					14.56
5(6)-Chloro	A	48.0	295–297¶	15.20	15.35
					15.35
5(6)-Bromo	B	23.0	300–301	12.67	12.58
					12.58
5(6)-Iodo	B	28.6	361 (dec.) (soft. 208)	9.87	9.77
					9.82
Benzo [g]	B	35.3	290 (dec.)‖	13.99	13.60
					13.68

* Uncorrected for stem emergence.

† Analyses by the micro-analytic section, Parke, Davis and Company.

‡ Lellman (4) who prepared this compound by heating o-phenylene di-(thiocarbamide) at 120–130°, reports m.p. 290 and an 80% yield. Billeter and Steiner (4) gives the m.p. as 298° when the thiol is prepared from o-phenylenediamine and thiophosgene and crystallized from alcohol.

§ Lellman (4) found m.p. 284°; Billeter and Steiner (7), m.p. 285.

¶ Fischer and Limmer (8), m.p. 270°.

‖ From 1,2-diaminonaphthalene. Ref. 9, gives the m.p. as above 300°

TABLE 2

Imidazoles and Benzimidazoles

COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD (per cent)	AVERAGE DOSE (mg/kg/day)	BODY WEIGHT GAIN (gm/day)	THYROID WT. (mg/100 gm. rat)	THYROID IODINE (mg. per cent)	ESTIMATED ACTIVITY THIOURACIL = 100
2-Imidazoline-2-thiol	$NH-CH_2-CH_2N=C-SH$	3 6*	0.01 0.10	11 73	3.3 -0.7	9.2 18.1	11.3 8.5	63
1,1,5,5-Tetramethyl-2-imidazoline-2-thiol	$NH-C(CH_3)_2-C(CH_3)_2N=C-SH$	3 3	0.01 0.10	12 94	3.5 2.0	10.7 20.4	25.4 13.1	10
1-(α-Methylbenzyl)-2-imidazoline-2-thione	$C_6H_5CH(CH_3)N-CH_2CH_2NHC=S$	3 3	0.01 0.10	9.3 94	1.8 1.9	7.0 8.2	61.6 40.0	0
S-(p-t-octylphenoxyethoxy ethyl)-2-imidazoline-2-thiol hydrochloride	$C_8H_{17}C_6H_4O(CH_2)_2O(CH_2)_2$ ·HCl $NHCH_2CH_2-N=C$ — S	3 3	0.01 0.10	12 103	3.8 2.8	6.2 7.8	60.5 32.2	1
2,2'-Ethylenebis-(2-imidazoline-1-ethanol)	$NCH_2CH_2N=C-(CH_2)_2-C=NCH_2CH_2N$ CH_2CH_2OH $HOCH_2CH_2$	3 3	0.006W 0.06 W	11 102	3.1 3.5	7.2 5.5	44.0 44.7	0

Compound		Dose	No.				
Benzimidazole-2-thiol (116)		0.0003	6	0.3	3.2	6.5	51.3
		0.0006	3	0.7	2.8	7.3	39.4
		0.001	6	1.1	3.1	8.7	30.1
		0.003	3	3.3	3.0	10.7	14.9
		0.006	3	0.4	3.3	11.1	13.0
		0.01	6	11	3.2	23.1	3.6
		0.03	3	28	2.5	33.8	1.6
		0.055	6	50	2.3	39.2	1.9
		0.10	6	79	1.4	43.6	3.7
		0.50†					
5(6)-Chlorobenzimidazole-2-thiol (44)		0.0003	3	0.35	3.6	7.8	52.2
		0.0006	3	0.64	3.0	7.1	74.5
		0.001	6	1.1	3.3	7.6	64.4
		0.003	6	3.4	3.4	6.7	34.1
		0.0055	6	5.5	3.0	8.4	20.9
		0.01	6	11	3.4	10.1	15.0
		0.04	6	35	2.5	19.2	3.9
		0.055	6	57	2.6	25.0	2.5
		0.10	6	76	1.2	26.8	2.9
5(6)-Bromobenzimidazole-2-thiol (27) 5(6)—Br—C6H3N=C(SH)NH		0.003	3	3.8	6.1	48.9	
		0.01	3	3.3	9.3	18.6	
		0.05	3	2.9	20.9	2.0	
5(6)-Iodobenzimidazole-2-thiol (11) 5(6)—I—C6H3N=C(SH)NH		0.003	3	3.4	6.5	60.6	
		0.01	3	4.0	7.3	29.1	
		0.05	3	3.6	12.7	12.2	
5(6)-Methoxybenzimidazole-2-thiol (14) 5(6)—CH3O—C6H3N=C(SH)NH		0.001	3	2.4	7.4	34.2	
		0.005	3	3.4	10.6	59.2	
		0.01	3	3.1	8.8	58.2	
		0.05	3	3.4	16.3	9.5	

Structures: Benzimidazole-2-thiol (positions 1,2,3,4,5,6,7 with N—H, C—SH, N). 5(6)-Chlorobenzimidazole-2-thiol (with Cl substituent).

TABLE 2—*Concluded*

COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD	AVERAGE DOSE	BODY WEIGHT GAIN	THYROID WT.	THYROID IODINE	ESTIMATED ACTIVITY THIOURACIL = 100
			per cent	*mgm./kg./day*	*gm./day*	*mg./100 gm. rat*	*mg. per cent*	
5(6)-Ethoxybenzimidazole-2-thiol	5(6)—C₂H₅OC₆H₃N=C(SH)NH	3 / 3	0.01 / 0.10	11 / 114	2.7 / 2.3	7.1 / 9.7	49.1 / 27.9	2
1-Methylbenzimidazole-2-thiol	C₆H₄N=C(SH)N(CH₃)	6 / 9	0.01 / 0.10	12 / 117	3.8 / 3.6	7.2 / 9.4	38.2 / 18.3	3
1-n-Butylbenzimidazole-2-thiol	C₆H₄N=C(SH)N(C₄H₉)	3 / 3	0.01 / 0.10	11 / 115	2.2 / 2.3	7.3 / 16.5	48.5 / 9.2	7
5(6)-Methylbenzimidazole-2-thiol	5(6)-CH₃C₆H₃N=C(SH)NH	3 / 3	0.01 / 0.10	10 / 89	2.2 / 2.1	8.4 / 22.9	60.3 / 6.6	12
Benzo[g]bonzimidazole-2-thiol (1,2-Naphthimidazole-2-thiol)	1,2—C₁₀H₆NHC(SH)=N	3 / 3	0.01 / 0.10	10 / 115	4.0 / 3.5	6.9 / 11.5	64.5 / 16.3	4
2-Aminobenzimidazolo	C₆H₄N=C(NH₂)NH	3 / 3	0.01 / 0.10	12 / 112	3.9 / 2.4	7.5 / 9.3	30.1 / 12.0	6
2-Amino-5(6)-chlorobenzimidazole	5(6)—ClC₆H₃N=C(NH₂)NH	3 / 3	0.01 / 0.10	12 / 109	4.4 / 3.2	6.9 / 7.7	91.1 / 46.3	0
2-Amino-5(6)-methylbenzimidazolo	5(6)—CH₃C₆H₃N=C(NH₂)NH	3 / 3	0.01 / 0.10	12 / 100	3.1 / 1.4	7.3 / 8.7	49.8 / 36.3	1

Compound	Formula	No. of rats	Dose (%)						Goiter
2-Benzenesulfonamido-benzimidazole	$C_6H_4N=C(NHSO_2C_6H_5)NH$	3 / 3	0.01 / 0.10	12 / 118	2.9 / 2.9	4.3 / 3.8	6.4 / 7.1	62.9 / 60.4	0
Di-benzenesulfonyl-2-amino-benzimidazole	$C_6H_4N=CN(SO_2C_6H_5)_2NH$	3 / 3	0.01 / 0.10	12 / 127	2.1 / 1.5	3.0 / 2.9	8.3 / 9.9	49.1 / 70.3	0
2-Phenyliminobenzimidazole	$C_6H_4N=C(NHC_6H_5)NH$	3 / 3	0.01 / 0.10	13 / 122	3.9 / 3.2	3.5 / 3.9	6.7 / 7.2	54.6 / 60.7	·
Dioctyl-2-guanidobenzimidazole dihydrochloride	$C_6H_4N=C(NHC(=NH)NH)N \cdot 2CH_3CO \cdot 2HCl$	3 / 3	0.05 / 0.50	12 / 103	–4.0 / 21.2	2.9 / 4.9	6.3 / 4.0	50.8 / 70.3	0
5(6)-Methyl-2-guanidobenz-imidazole	$5(6)-CH_3C_6H_3N=C(NHC(=NH)NH)NH$	11 / 111	0.01 / 0.10	10 / 90	1.9 / –4.0	3.9 / 3.2	0.1 / 0.8	72.1 / 52.0	0
Benzimidazole	$C_6H_4N=CHNH$	0 / 6†	0.01 / 0.10	11 / 62	3.7 / 3.7	7.9 / 0.2	49.0 / 41.4	Tr.	0
Phenylthiourea	$C_6H_5NHCSNH_2$	65 / 503	0.01 / 0.10	3.9 / 3.2	3.7 / 3.7	8.5	14		
Benzothiazole-2-thiol	$C_6H_4N=C(SH)S$	12 / 112	0.01 / 0.10	3.5 / 3.4	0.6 / 0.5	7.9 / 0.2	44.8 / 29.2	2	
6-Chlorobenzothiazole-2-thiol	$6-ClC_6H_3N=C(SH)S$	11 / 111	0.01 / 0.10	0.4 / 7.7	6.6 / 0.7	48.5 / 45.7	0	0	
4-Methoxybenzothiazole-2-thiol	$4-CH_3OC_6H_3N=C(SH)S$	12 / 117	0.01 / 0.10	3.4 / 3.2	43.0 / 53.3	0			
4,5-Diphenylimidazole	$NH-C(C_6H_5)=C(C_6H_5)N=CH$	3 / 3	0.01 / 0.10	3.0 / 1.8	8.5 / 8.1	69.0 / 67.0	0		

* Two rats dead on eighth day. Average weight gain based on surviving rats.
† All rats dead by seventh day.
‡ One rat died on fifth day of treatment.
W: Administered in drinking water.

Method A was used in those cases in which it was desirable to isolate the intermediate diamine for other purposes. The diamines were prepared by reduction of the corresponding nitroaniline. This method required less manipulation than the procedure described by Lellman (4). In those instances in which it was not desirable to isolate the notoriously unstable o-phenylenediamines, Method B was employed. The yields reported for the second method are therefore based on the substituted o-nitroaniline used as starting material. For example, 5(6)-iodo-benzimidazole-2-thiol was prepared from 4-iodo-2-nitro-aniline (5). The latter (32.5 gm.) was reduced in 400 ml. of absolute alcohol with hydrogen under 50 pounds pressure using platinum oxide catalyst. After the reduction was completed, the catalyst was removed by filtration and the resulting solution of 4-iodo-2-amino-aniline was refluxed for 18 hours with 30 ml. of carbon disulfide and sufficient 50% sodium hydroxide solution to make it strongly basic (Hydrion paper). The reaction mixture was evaporated to dryness on the steam bath and the residue treated with 300 ml. of water. The pH of the resulting mixture was adjusted to 10–11 and the insoluble material removed by filtration. Acidification of the filtrate with concentrated hydrochloric acid yielded a light tan product which was collected and purified by dissolving it in alcohol, decolorizing with charcoal and precipitating it from the hot solution with water. The process was repeated using isopropyl alcohol but two precipitations from aqueous alkali using carbon dioxide as the neutralizing agent, were required to obtain a light yellow product. The yield was 9.7 grams or 28.6% of theory. Some loss undoubtedly results from the long purification procedure which was believed necessary to remove undesirable colored by-products. The benzimidazole-2-thiols are all soluble in diluted sodium hydroxide solution. Iodine T.S.-U.S.P. is decolorized by an alkaline solution ol benzimidazole-2-thiol.

The synthesis of the 2-amino- and 2-guanidobenzimidazoles described in table 1 will be the subject of another communication. The 4-methoxy-benzothiazole-2-thiol and the 2-imidazoline-2-thiol were supplied by the Eastman Kodak Company and the other imidazolines with the exception of S-(p-t-octylphenoxyethoxyethyl)-2-imidazoline-2-thiol hydrochloride were obtained from Carbide and Carbon Chemicals Corporation. The phenoxyethoxyethyl derivative was prepared by Dr. George Rieveschl. The benzothiazole-2-thiol and 6-chloro-benzothiazole-2-thiol were secured from Dow Chemical Company. The 4,5-diphenylimidazole was prepared by the method of Pinner (6).

RESULTS. Experimental details and the method used to estimate antithyroid activity are described in the previous publication (1). Table 2 contains the essential information from which these calculations were made.

Imidazole Derivatives. It may be observed from table 2 that 2-imidazoline-2-thiol with an activity of 63 has approximately twice the antithyroid potency of ethylthiourea which has a value of 35 (1). However, it is definitely more toxic than ethylthiourea as judged by the average daily weight gain during he 10 day test period. These data show that ring closure as in the case of phenyl-thiourea, results in enhanced goitrogenic activity at least within the dose range below toxic levels. It should be pointed out that phenylthiourea appeared to be more toxic than benzimidazole-2-thiol whereas the reverse was the case with ethylthiourea and 2-imidazoline-2-thiol.

C-Tetramethylation of 2-imidazoline-2-thiol resulted in marked loss of activity as was also the case when one nitrogen was substituted by an α-methylbenzyl group. Only one compound with a substituent on the sulfur atom was included in this group. Although the p-t-octyl-phenoxyethoxyethyl radical imparted great surface activity to the parent compound, the antithyroid properties of the latter were almost lost. It was concluded on the basis of these findings

that substitution resulted in decreased activity, consequently no other derivatives were studied, particularly in view of the activity found in thiazoline-2-thiol (1). The comparative effectiveness of these isologs will be discussed in another communication which will disclose the activity of several thiazolines tested during the course of this study.

Benzimidazoles. Substitution of chlorine, bromine or iodine in the 5-position of benzimidazole-2-thiol resulted in decreased antithyroid activity without

COMPARATIVE DOSE-RESPONSE CURVES

2-THIOURACIL
BENZIMIDAZOLE-2-THIOL
5(6)-CHLORO BENZIMIDAZOLE-2-THIOL
5(6)-BROMO BENZIMIDAZOLE-2-THIOL
5(6)-IODO BENZIMIDAZOLE-2-THIOL
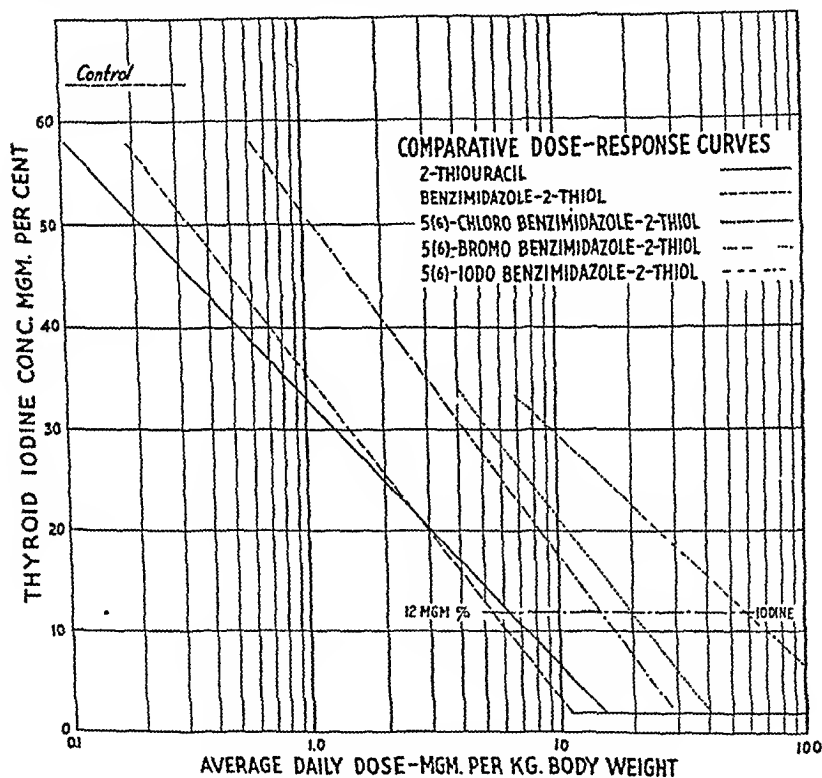
Fig. 1

effecting relative toxicity of the parent compound. It will be noted that the activity decreased by half with each substitution in the order: benzimidazole-2-thiol, 5(6)-chloro-, 5(6)-bromo-, 5(6)-iodo-benzimidazole-2-thiol. It was our intention to introduce radioactive iodine into the molecule had the activity of the iodo derivative warranted an extensive study of tissue distribution and excretion. However, this study was not justified on the basis of the observed activity. The dose-response curves for these four compounds and for thiouracil are shown in figure 1. The similarity of slopes of these lines, as plotted on

semi-logarithmic paper to those described in our first paper, emphasizes our conclusions that, in general, dose-response gradients of compounds studied fall into a definite pattern.

Introduction of alkoxyl and alkyl groups into the benzenoid ring of benzimidazole-2-thiol also reduced the activity. Alkylation in the 1-position decreased the effectiveness as did substitution of an —NH₂ group for the —SH group. Moreover, the many derivatives of 2-amino-benzimidazole were devoid of activity. Replacement of one N atom in benzimidazole-2-thiol by sulfur led to diminished activity as shown by the tests with benzothiazole-2-thiol and its two derivatives. Benzimidazole and 4,5-diphenylbenzimidazole are inactive.

In view of the above results and the pronounced antithyroid properties of benzimidazole-2-thiol in the rat, this compound is recommended for further study in other animal species.

SUMMARY

Twenty-six compounds belonging to the imidazole or benzimidazole groups have been tested for antithyroid activity. All of these are less active than benzimidazole-2-thiol. Certain relationships between chemical structure and antithyroid activity have been investigated and are discussed in the light of experimental data obtained.

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THE EXPERIMENTAL BASIS FOR THE QUANTITATIVE CHEMO-THERAPY OF B. NOVYI IN MICE WITH A COMPARISON OF ACTION OF PENICILLIN AND DICHLORPHENARSINE HYDROCHLORIDE

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Of all the actions of penicillin perhaps the most surprising is that against spirochetes. If nothing else penicillin has conclusively demonstrated that potent spirocheticidal substances need not contain a heavy metal. Moreover it is unlikely that penicillin is the only nonmetallic compound which will show such action. Therefore there is need for a procedure whereby a large number of compounds can be screened for spirocheticidal activity. Although syphilis is the most important spirochetal disease, it does not lend itself well to such a program, because of the size of the host and prolonged course of the experimental disease. In as far as is known most agents affecting one type of spirochete have some action on others. For this reason we have carried out studies to see if *B. novyi* (1, 2)[1] infections in mice might be used for a rapid screening test. This organism was selected because it has been carried in mice for a considerable period of time, and is known to produce a virulent disease which we believed could be easily standardized. It is supposed that this organism is the same as causes one type of relapsing fever in man.

In most instances previous experiments on relapsing fever have been carried out in such a manner that it has been impossible to draw conclusions as to the quantitative action of penicillin. It seemed important to obtain certain basic information on the course of untreated *B. novyi* infections in white mice before proceeding to the actual chemotherapy experiments.

METHODS White mice of 17 to 24 grams were used throughout. All inoculations were made intraperitoneally with appropriate dilutions of blood obtained by cardiac puncture from unanesthetized donor mice. Subinoculation was carried out at two day intervals.

In most animals the course of the infection was followed by the study of "thin film" blood smears stained with Giemsa stain according to the standard method. On occasion dark field and "thick film" studies were also made. Thin film smears were selected for routine work because these permitted actual enumeration of parasites and made possible the handling of larger groups of mice. In heavy, or moderately heavy infections red cells and spirochetes were counted until at least 50 organisms had been seen. In light infections parasites were counted by fields until a total of 10,000 red cells had been observed or until 50 organisms had been seen. All counts were expressed as number of parasites per 10,000 red cells.

All inoculations were made between 10 A.M. and 12 M., and blood films were taken at this same time each day.

Hemoglobin concentration was determined by the standard technique of converting hemoglobin to alkaline hematin and determining the intensity of the color with a photo-electric colorimeter.

THE COURSE OF UNTREATED INFECTIONS. It was immediately evident that the dose of parasites administered had a considerable affect on the course of untreated infections. A number of experiments were carried out to determine the dose of parasites producing the most suitable infection for chemo-

TABLE 1

Course of untreated B. novyi infections in white mice. Effect of variations in number of parasites in the inoculating dose

Ten mice used at each dosage level

DAYS AFTER INOCU-LATION	INOCULATING DOSE—PARASITES PER KILO			
	1×10^{10}	1×10^{9}	1×10^{8}	1×10^{7}
Mean parasite count per 10,000 red cells				
0*	3 ±1.0			
1	878 ±227	101 ±64	1.6 ±0.6	1
2	8,146 ±1533	1,490 ±393	215 ±56	3.5 ±1.2
3	7,594 ±1261	5,197 ±765	1481 ±362	395 ±57
4	1,556 ±1027	3,010 ±1132	1644 ±903	248 ±203
5	<1	<1	<1	<1
6	<1	<1	<1	<1
Hemoglobin concentration—grams per cent				
1	16.6 ±0.59	16.8 ±0.35	16.4 ±0.54	16.3 ±0.43
2	10.7 ±0.55	11.6 ±0.33	11.0 ±0.44	11.6 ±0.26
3	10.2 ±0.65	9.7 ±0.47	11.4 ±0.42	12.8 ±0.43
4	7.4	7.7 ±0.50	9.6 ±0 41	10.7 ±0.64
5		9.1 ±0.53	10.6 ±0 50	12.1 ±0.48
7		12.4 ±0.63	12 9 ±0.58	13.5 ±0.58
9		12 3 ±0.48	12.8 ±0.07	13 0 ±0.66
Deaths in 14 days	7/10	2/10	1/10	0/10

* Slides on 0 Day taken 6 hrs. after inoculation.

therapeutic studies. The results of one such experiment are presented in table 1. Forty mice were divided into groups of 10, and inoculated intraperitoneally with pooled blood from the same donor mice, with dose ranging from 1×10^{7} to 1×10^{10} parasites per kilo. Regardless of the number of organisms inoculated, the acute phase of the disease, (as judged by parasites in the peripheral blood) lasted four days. The peak of parasitemia being reached on the 2nd and 3rd day. By the 5th day spirochetes could no longer be found in thin smears, regardless of parasite dosage. Variations in size of inoculum affected only the peak of parasitemia and survival of the animals. Injection of 1×10^{10} spirochetes per kilo produced an infection in which there was about

one organism for each red cell at the peak of parasitemia, and approximately 70 per cent of the mice died. Injection of 1×10^7 spirochetes per kilo ordinarily killed no animals and produced a peak of parasitemia which was about $\frac{1}{10}$ that of the larger inoculum.

The degree of anemia produced by *B. novyi* infections in mice was also related to the number of organisms inoculated, and was roughly proportional to the number of organisms seen in the peripheral blood. The decrease in hemoglobin followed the parasite curve by about 24 hours, and was most marked on the 4th day. From a standpoint of parasite enumeration this was fortunate as it meant that the determination of ratio of spirochetes to red cells up to the peak of parasitemia was a fairly accurate indication of the total number of organisms present in the peripheral blood.

It was apparent from a number of experiments such as presented in table 1 that inoculation with 1×10^{10} organisms per kilo gave the most suitable infection for chemotherapeutic experiments. This dosage of parasites produced a high percentage of deaths and the course of the infection was constant enough to permit a quantitative evaluation of chemotherapeutic agents.

In each experiment described later in this paper we included a group of at least 5 mice which served as untreated controls. In all instances these received the so-called standard inoculation of 1×10^{10} organisms per kilo. A statistical analysis of the results obtained in 100 such untreated mice permitted a base line from which the effect of drugs upon the infection could be evaluated. Parasite counts were made routinely on the 2nd and 3rd day after inoculation. Since this infection is characterized by a sharp peak we selected the highest count observed and used this as a criterion of degree of infection in each mouse. Thus in the 100 mice used in 20 experiments, conducted at approximately weekly intervals, the mean of the highest parasite counts was 11,705 organisms per 10,000 red cells. This mean had a standard error of ± 369. Figure 1-A gives the distribution of parasite peaks in these 100 mice and it is to be noted that they are normally distributed around the mean.

Sixty three per cent of the animals had their highest parasite count on the second day after inoculation whereas, 37% had their peak on the third day. In this group of untreated controls 70.0% died within two weeks after inoculation, the distribution of day of these deaths is indicated in figure 1-B from which it is observed that roughly 80% of those animals which died, did so before the seventh day.

THE EFFECT OF CHEMOTHERAPEUTIC AGENTS UPON STANDARDIZED INFECTIONS. Lourie & Collier (3) were probably the first to demonstrate the effect of penicillin on spirochetes. These observations have been confirmed by a number of investigators (4, 5, 6, 7) using a variety of species of parasites. With the exception of the work by Eagle and Magnuson (5) little attempt has been made to standardize the infection or to administer drugs in such a way as to permit a quantitative assay of the action of penicillin.

Most investigators have postponed treatment for at least 24 hours or until heavy infection has been established. Since the acute phase of relapsing fever runs a relatively short course it is difficult to differentiate between a drug effect
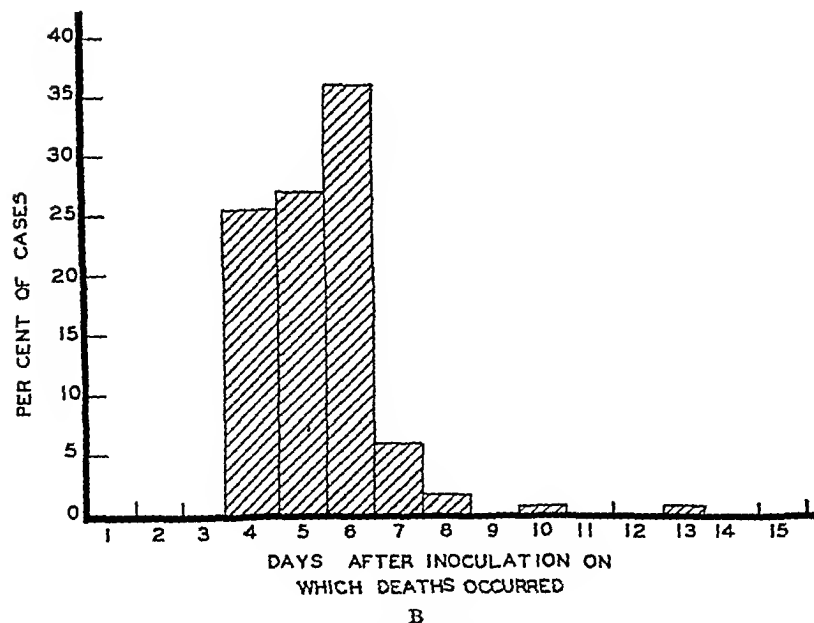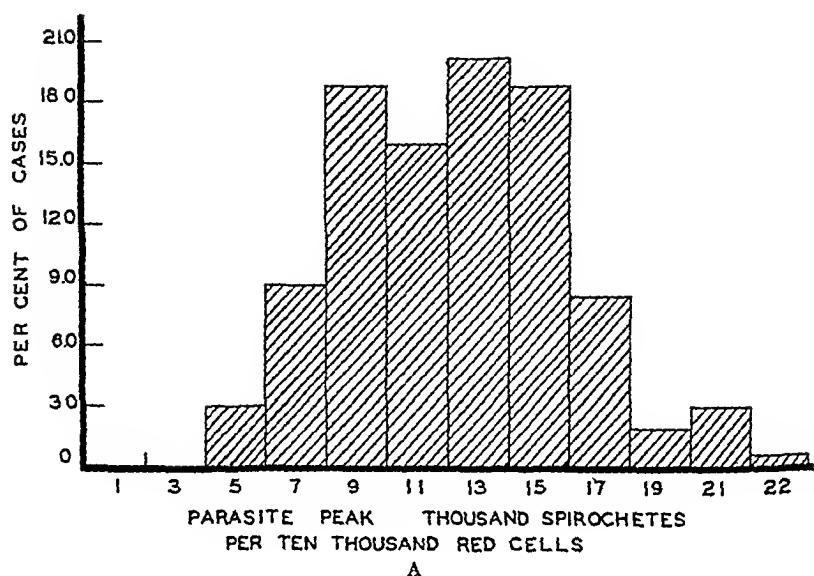
FIG. 1. COURSE OF UNTREATED B. NOVYI INFECTIONS IN 100 WHITE MICE
Inoculated intraperitoneally with 1×10^{10} spirochetes per kilo.

and spontaneous remission, if one delays treatment too long. We designed our experiments so as to obtain the greatest possible difference between untreated controls and successfully treated animals. For this reason therapy was instituted as soon as possible. In the case of parenteral administration this was within 1 hr. after inoculation. Where drug has been administered in the diet, treatment was started 18 hrs. prior to inoculation. In a sense our experiments might best be described as suppressive rather than therapeutic.

Five samples of penicillin were compared for their spirocheticidal activity:[2]

Sample No. 1. Crystalline sodium penicillin G assaying 1650 Oxford units per milligram. Since this was the only pure sample except for the methyl ester available to us it was used as a standard of reference.

Sample No. 2. Commercial sodium penicillin Squibb (lot no. 32931), assaying 913 Oxford units per milligram. It was probably a mixture of Penicillin G and other penicillins, including some F.

Sample No. 3. Pilot plant lot no. 860, which assayed 810 Oxford units per milligram. The exact composition of this sample was uncertain except that it contained almost no penicillin G.

Sample No. 4. Commercial calcium penicillin Squibb (lot no. 3312-1) containing 927 units per milligram. Probably a mixture of G and other penicillins.

Sample No. 5. Methyl ester of Penicillin G prepared from sample No. 1. By *in vitro* tests this material had an activity of 20–40 units per mgm. Dosage of this sample was calculated on the basis of units it would have had if it had been quantitatively hydrolyzed in the body to a dissociable salt of free penicillin G.

These samples of penicillin and dichlorphenarsine hydrochloride were compared for their activity against *B. novyi* in three types of experiments, differentiated only by method of treatment.

1) *Frequent Subcutaneous Injections:* Treatment was started immediately after inoculation. The total daily dose was split up into 5 doses & administered at 4 hour intervals (actual injection schedule 7 A.M., 11 A.M., 3 P.M., 7 P.M., 11 P.M.). Water solutions of drugs were used except in the case of the water insoluble methyl ester which was suspended in 2.5% starch. Each injection was made subcutaneously on the back, in a volume of 0.2 cc. for a 20 gram mouse. Treatment was continued for a total of 9 doses.

2) *Single Daily Injections:* The purpose of these experiments was to detect any possible differences in rate of excretion of the various types of penicillin. Treatment was carried out as described above except that the entire daily dose was given within 1 hr. after inoculation and repeated 24 hrs. later.

3) *Drug-Diet:* The drug was mixed with powdered Purina Dog Chow in a flour mill, and given to the experimental groups 18 hrs. before inoculation.

TABLE 2

Protocol of Experimental Series No. 29, March 5, 1945. Comparison of commercial sodium penicillin and calcium penicillin, administered subcutaneously every four hours

MOUSE NO	TOTAL DAILY DOSE	SPIROCHETE COUNTS PER 10,000 RED CELLS		HIGHEST PARASITE COUNT PER CENT UNTREATED CONTROLS	DAY OF DEATH TO 14 DAYS
		2nd. Day	3rd Day		
Commercial calcium penicillin					
	units per kilo				
1	1560	3,470	11,600	107.00	
2	1560	4,620	13,100	120.50	
3	1560	3,850	10,500	96.40	
4	1560	752	13	6.90	
5	1560	3,170	10,700	98.50	
1	3125	6,000	9,730	89,50	
2	3125	0	2	0.02	
3	3125	1,640	784	15.10	
4	3125	4,390	3,570	40.40	
5	3125	1,750	4,730	43.50	
1	6250	1,000	2,860	26.30	
2	6250	106	100	1.00	
3	6250	130	5	1.20	
4	6250	295	24	2.70	
5	6250	5	0	0.05	
1	12,500	3	0	0 03	
2	12,500	15	0	0 14	
3	12,500	1	0	0 01	
4	12,500	0	0	0 00	
5	12,500	0	0	0.00	
Commercial sodium penicillin					
1	1560	2,230	12,400	114.00	
2	1560	2,750	6	25.20	
3	1560	6,240	11,700	107.00	
4	1560	5,980	12,800	117 80	
5	1560	7,400	14,800	135 00	
1	3125	2,500	13,300	121 80	
2	3125	1,490	1,980	18.10	
3	3125	530	12	4 85	
4	3125	1,530	449	14.00	
5	3125	400	3,260	29.80	
1	6250	952	1,090	10.00	
2	6250	20	1	0.18	
3	6250	583	1,970	18.10	
4	6250	1,800	1,700	16 50	
5	6250	1,260	1,430	13 20	

TABLE 2—*Concluded*

MOUSE NO.	TOTAL DAILY DOSE	SPIROCHETE COUNTS PER 10,000 RED CELLS		HIGHEST PARASITE COUNT PER CENT UNTREATED CONTROLS	DAY OF DEATH TO 14 DAYS
		2nd. Day	3rd. Day		
Commercial sodium penicillin—*Continued*					
	units per kilo				
1	12,500	0	1	0.01	
2	12,500	0	1	0.01	
3	12,500	0	0	0.00	
4	12,500	3	0	0.03	
5	12,500	69	2	0.69	
Untreated controls					
1		10,200	9,720	Mean of	6
2		10,600	6,610	highest	
3		13,300	6,250	counts	
4		10,900	6,070	10,886 =	
5		9,430	2,030	100%	7

Mice, usually in groups of five were placed in a cage and supplied with a common food cup, from which an excess supply of drug-diet was available. No attempt was made to determine the drug intake of individual mice; rather the average for the entire group was calculated. Treatment was continued for 4 days. Calcium penicillin diets prepared in this way were stable for at least 3 weeks without loss of potency.

Because of the conditions under which the experiments were carried out, the maximum number of animals which could be studied at one time was approximately 40. This permitted the comparison of only two preparations at any one time. Ideally the standard of reference crystalline penicillin G, should have been used in each series of tests. Limitations on the supply of this preparation prevented such a procedure. For a final comparison of the activity of the various preparations it was therefore necessary to combine the results of a number of experiments carried out over a period of several months.

Table 2 gives the protocol of a typical experiment in which two preparations of penicillin were compared. Ordinarily four doses of each preparation were used; each being administered to a group of at least 5 mice. Parasite counts were made on the second and third day after inoculation, and the highest taken as a criterion of the intensity of infection produced. Since there was some variation in virulence of the parasite from week to week, counts were expressed as a per cent of the mean peak of parasitemia observed in untreated controls. Such a calculation made the results obtained from experiment to experiment more nearly comparable.

Table 3 presents a composite summary of all the comparative experiments in which the various preparations were administered at four hour intervals. As can be seen from figure 2 there is a straight line relationship between dosage-

TABLE 3

Comparison of various penicillin preparations and dichlorphenarsine hydrochloride on
B. novyi infections treated every four hours 5 times daily for 2 days

| TOTAL DAILY DOSE	NO. MICE	AVERAGE PARASITE PEAK. PER CENT OF UNTREATED CONTROLS	MORTALITY IN 14 DAYS	AVERAGE DAY OF DEATH
Crystalline sodium penicillin G				
units per kilo/day				
1560 (0.97 mgm.)	34	70.3 ± 7.9	7/34	4.5
3125 (1.94 mgm.)	35	51.0 ± 6.0	15/35	6.5
6250 (3.87 mgm.)	33	24.2 ± 5.0	3/33	6.0
12,500 (4.75 mgm.)	34	6.2 ± 2.5	1/34	
25,000 (15.0 mgm.)	10	<0.01	0/10	
Pilot Plant Lot 860				
1560	10	65.0 ±11.8	3/10	4.2
3125	10	46.2 ± 8.0	2/10	11.0
6250	10	29.4 ±10.4	2/10	6.0
12,500	9	16.2 ± 8.1	1/9	
25,000	10	<0.01	0/9	
Commercial sodium penicillin				
1560	19	65.9 ± 8.0	7/19	6.8
3125	15	32.7 ± 7.5	6/24	6.5
6250	23	4.1 ± 1.2	3/23	8.0
12,500	25	0.61 ± 0.07	0/25	
25,000	20	<0.01	0/20	
Commercial calcium penicillin				
1560	10	79.5 ±17.8	4/10	8.0
3125	15	63.5 ± 5.1	1/15	
6250	15	30.8 ±10.0	0/15	
12,500	15	8.1 ± 3.8	0/15	
25,000	5	0.02	0/5	
Dichlorphenarsine hydrochloride				
0.62 mgm./kg.	10	73.0 ± 8.2	7/10	5.1
1.25 mgm./kg.	10	75.3 ± 4.5	7/10	5.5
2.5 mgm./kg.	9	37.7 ± 8.7	1/9	
5.0 mgm./kg.	15	3.7 ± 2.9	0/15	
Methyl ester of penicillin G				
1560	25	72.8 ± 7.2	11/25	7.1
3125	25	49.2 ± 7.2	10/25	5.6
6250	23	33.7 ± 6.5	5/23	5.8
12,500	30	16.4 ± 4 5	6/30	9.7
25,000	10	<0.01	0/10	
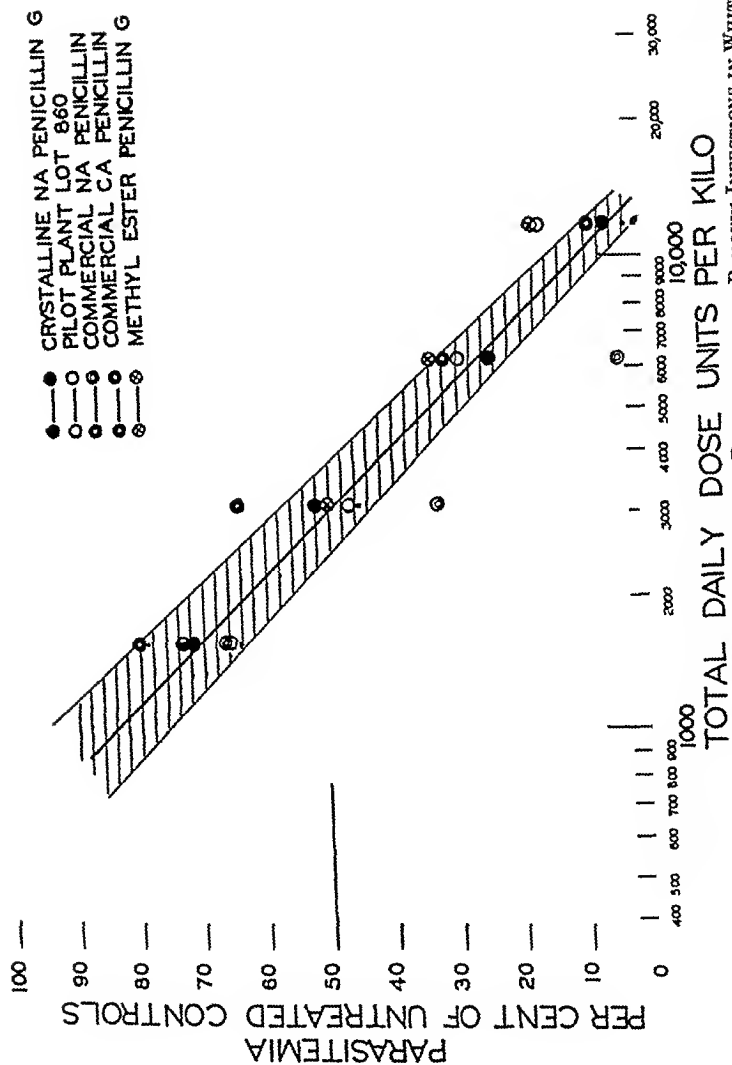
FIG. 2. SUMMARY OF EFFECT OF FIVE DIFFERENT PENICILLIN PREPARATIONS ON B. NOVYI INFECTIONS IN WHITE MICE Drugs administered subcutaneously at four hour intervals.

response, if dosage is plotted logarithmically and parasite counts are plotted arithmetically. An estimated ED_{50} can then be read off the curve. The ED_{50} for each preparation thus determined is presented in the 3rd column of table 6.

The question immediately arises as to the error of the ED_{50} thus determined, and more especially whether the differences between the various penicillin preparations were significant. Since there is a question of whether experimental data of this kind can be treated in the same way as data of the all-or-none type, we did not attempt an elaborate statistical analysis of it. However, in order to obtain some estimate of the error involved, the dosage-response curve of crystalline sodium penicillin G has been drawn in Figure 2 with one standard error on either side of the mean. The enclosed area has been cross hatched and represents the area within which one might expect the dosage-response curve of the standard drug to fall 2 out of 3 times. From such a graphic representation it can be estimated that the ED_{50} of crystalline sodium penicillin G might vary from 2300 to 3900 units per kilo, hence it is obvious that differences between the estimated ED_{50}'s of the penicillin preparations are not highly significant.

Such a conclusion is further strengthened if one refers to table 2 in which the commercial sodium salt of penicillin was compared to the commercial calcium salt. In the final estimation of the ED_{50} these two preparations differed most widely. However when these two preparations were used in the same experiment as shown in table 2, no difference in activity can be detected.

As crystalline sodium penicillin G was the only chemically pure penicillin salt used, it was the only one in which dosage could be expressed in units of weight. It was therefore the only one with which dichlorphenarsine hydrochloride could be compared. Inspection of the data in table 3 and a graphic estimation of the ED_{50} indicates that milligram for milligram dichlorphenarsine hydrochloride has about the same activity as pure sodium penicillin G.

Table 4 presents composite data of experiments in which the effect of 3 samples of penicillin and dichlorphenarsine hydrochloride on the standardized infection were compared by the method of single daily dose treatment. Plotting this data on semi-logarithmic paper as described above permitted an estimation of the ED_{50} for each preparation. These were tabulated in the 4th column of table 6. When penicillin is administered in this way, approximately 4 times as much drug is required to produce an effect as when it is administered by repeated injections at 4 hour intervals. Due to the smaller number of mice used in the single daily dose experiments, it is more difficult to compare the activity of compounds. However it is doubtful if the 3 preparations of penicillin studied, differed quantitatively.

A comparison of the activity of four samples of penicillin administered in the diet is presented in table 5. Estimations of ED_{50} from this data is given in the 2nd column of table 6. It is of interest that therapeutic effects can be obtained by the drug-diet method with amounts of penicillin which are only 3–4 times that required when the drug is administered by means of frequent subcutaneous injections. Similar observations have been made by Libby (8) in the treatment of bacterial infections in mice. As in the case of the previous

two types of experiments no difference in activity between the various salts of penicillin was noted.

The methyl ester of Penicillin G represents a special case. Impure preparations reported to be esters of penicillin were first studied by Hobby & Meyers (9) who concluded they were more active by mouth than the corresponding

TABLE 4

Comparison of effect of various penicillin preparations and dichlorphenarsine hydrochloride on B. novyi infections

Treatment 1 dose daily

DAILY DOSE	NO MICE	AVERAGE PARASITE PEAK PER CENT OF UNTREATED CONTROLS	MORTALITY IN 14 DAYS	AVERAGE DAY OF DEATH
Crystalline sodium penicillin G				
units per kilo/day				
12,500 (7 75 mgm.)	5	30 3 ± 8 8	1/5	
25,000 (15 5 mgm.)	5	8 6 ± 4 7	0/5	
50,000 (31 0 mgm)	5	1 5 ± 0 8	0/5	
100,000	4	1 9	0/5	
Commercial sodium penicillin				
6,250	5	74 1 ± 6.2	1/5	
12,500	15	69 2 ±12 7	3/15	5.1
25,000	10	10.6 ± 4 9	0/5	
50,000	10	0.78 ± 0.7	0/5	
100,000	10	<0.01	0/5	
Pilot Plant Lot 860				
12,500	5	87.0 ± 9.9	2/5	5.0
25,000	5	4.1 ± 1.4	0/5	
50,000	5	13.3 ±11 8	0/5	
100,000	5	<0 01	0/5	
Dichlorphenarsine hydrochloride				
1 25	5	60 0 ±13 6	1/5	
2 5	5	67 0 ±10 7	0/5	
5 0	5	12 8 ±11 7	0/5	
10 0	5	<0 01	0/5	

freely dissociable salts. Although our experiments show the methyl ester to be about as active as the salts when it was administered parenterally, it was certainly less than $\frac{1}{10}$ as active when administered by drug-diet.

DISCUSSION. The experiments reported are not intended to answer the question as to ability of penicillin, or dichlorphenarsine to sterilize animals infected with *B. novyi*. As a matter of fact we have been unable to reinfect any of our animals in spite of treatment with the largest doses of penicillin described above. This is ordinarily taken as presumptive evidence that no cures had been effected.

TABLE 5

Comparison of effect of various penicillin preparations on B. novyi infections when administered by drug-diet procedure

DRUG-DIET CONCEN-TRATION	NO MICE	AVERAGE DRUG INTAKE	AVERAGE PARASITE PEAK PER CENT OF UNTREATED CONTROLS	MORTALITY IN 14 DAYS	AVERAGE DAY OF DEATH
Commercial sodium penicillin					
units per 100 gm.		*units/kg /day*			
4,166	10	5,317	88.7 ±13.9	8/10	6.2
8,333	15	15,694	13.5 ± 7.8	4/15	4.5
16,666	14	29,677	3.6 ± 3.6	1/14	
33,333	15	69,866	<0.01	0/15	
Crystalline penicillin G					
4,166	10	5,632	76.5 ±13 5	6/10	4.5
8,333	10	13,650	43.1 ±16.7	3/10	3.6
16,666	10	26,200	2.6 ± 2 4	0/10	
33,333	9	64,050	<0.01	0/10	
Commercial calcium penicillin					
4,166	10	4,695	83 3 ±21.1	4/10	7.9
8,333	10	12,510	22.4 ± 8.0	2/10	5.0
16,666	10	22,850	13.9 ± 7.9	2/10	3.0
33,333	10	64,000	<0.01	0/10	
Crystalline methyl penicillin G					
4,166	5	5,220	106.9 ±20.4	4/5	6.1
8,333	5	10,000	104 3 ±11 9	4/5	4.5
16,666	10	27,200	97 2 ± 9.7	7/10	4.7
33,333	10	32,000	63.5 ± 9.5	6/10	5.5
66,666	5	83,400	99.7 ± 6.3	5/5	4.8
133,333	5	187,000	92.8 ± 6 1	5/5	4.2

TABLE 6

Summary of effect of various penicillin preparations and dichlorphenarsine hydrochloride on B. novyi infections in white mice when administered by various routes

PREPARATIONS	ESTIMATED ED₅₀		
	Drug-diet	Subcutaneous 5 doses daily	Subcutaneous 1 dose daily
	units/kilo/day	*units/kilo/day*	*units/kilo/day*
Crystalline sodium penicillin G	9,800 (5.9)*	2,900 (1 8)*	10,300 (6 2)*
Commercial sodium penicillin	9,100	2,300	13,600
Pilot Plant Lot 860		2,750	15,400
Commercial calcium penicillin	8,600	3,450	
Crystalline methyl ester penicillin G	>187,000	3,150	
Dichlorphenarsine hydrochloride		1.9*	3 75*

* Milligrams per kilo per day.

Dunham & Rake (10) have reported evidence indicating that impure preparations of penicillin may contain substances other than penicillin itself, which have a spirocheticidal activity for *Spirocheta pallidum.* Our experiments do not allow one to conclude that any material other than penicillin was present in the preparations available to us, which had a chemotherapeutic action against *B. novyi.* To further study this point, samples of commercial sodium penicillin were inactivated by sodium hydroxide and tested for activity against *B. novyi* infections. Doses of such inactivated penicillin equivalent to 400,000 units per kilo per day were completely devoid of activity. The alkali inactivated penicillin was thus less than $\frac{1}{100}$ as active as the original penicillin from which it was made. If there was any material in our sample of commercial sodium penicillin, other than penicillin itself, which was spirocheticidal it must have been present in extremely low concentrations or it possessed properties of alkali instability resembling those of penicillin.

CONCLUSIONS

1. Experimental procedures for quantitative chemotherapeutic experiments with *B. novyi* in mice have been described.

2. Four preparations of penicillin varying in degree of purity and containing varying proportions of G and other penicillins have been compared for their spirocheticidal activity. On the basis of dosage in Oxford units no differences were noted between the four samples regardless of method of administration.

3. By parenteral injection the methyl ester of penicillin G is equal in activity to free penicillin G; however, orally the ester is less than $\frac{1}{20}$ as active.

4. When administered by frequent subcutaneous injection dichlorphenarsine hydrochloride is milligram for milligram equal to crystalline sodium penicillin G in activity against *B. novyi.*

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DISTRIBUTION AND FATE OF PENICILLIN IN THE BODY

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The distribution of penicillin in the animal body after injection, as well as the ultimate fate of that fraction not rapidly excreted, is inadequately known. This paper records information contributing toward a more complete understanding of these problems.

DISTRIBUTION IN BODY TISSUES. It is well recognized that only negligible amounts of penicillin enter the spinal fluid when the meninges are normal, but that there may be some penetration through inflamed meninges (1). Penicillin has also been demonstrated in ascitic, pleural and subcutaneous fluids (1), in articular fluids, and in the fetus (2), but detailed studies in other body tissues are scanty, except for the work of Struble and Bellows (3). These investigators studied the penetration of the agent into the various tissues of the eye of the dog with great care, and also, the distribution through other tissues in less detail. They found practically no penicillin in nerves, brain, dura, and bone marrow. A low concentration was present in voluntary and heart muscles, pancreas, adrenal, and spleen. Two or three times as much was found in liver, bile, lung, buccal mucosa, skin, and small intestine, and eight times as much in the kidney.

We made comparisons of the concentration of penicillin in the blood, and that in various tissues in 6 rats and 3 rabbits, one-half hour after the subcutaneous (rat) and intravenous (rabbit) injection of 1000 to 10,000 units[1] of penicillin. Rat tissues were homogenized in a Waring blendor in physiological salt solution and centrifuged. The rabbits were bled, the tissues removed and ground with sand, diluted with physiological salt solution and centrifuged. The penicillin was estimated in the supernatant fluid by the method of Wolohan and Cutting (4). The results are shown in table 1.

It is clear that, in both rats and rabbits, the brain contained only a negligible amount of penicillin, and the liver, heart, and striated muscle contained amounts approaching that in the blood. The penicillin content of the kidney is more variable than that of the other organs. In the case of the rabbit the variability is less than in the rat, and the content, apparently, significantly higher. Rabbit kidney contained almost 50 times the amount in the blood, while in only one instance was a concentration of this order found in rat kidney. The whole

[1] Due to the scarcity of penicillin when these tests were made, remnants of clinical solutions were used in many cases, without re-assay, but with use of the same solution in control and test runs on the same animal.

rat kidney was used, while cortical tissue was selected from the rabbit to decrease high results which contamination with urine might produce. Thus, the low quantities in the rat kidney suggest either that it is not concentrated there, as it may be in the rabbit kidney, or that in the rat kidney it is rapidly destroyed.

PRESENCE OF PENICILLIN IN SALIVA AND BILE. It has been shown (5) that penicillin is rapidly excreted in the bile, at a concentration persisting at a higher level than that in the blood, and that it also appears in the saliva. As this would be of importance if the proportion lost were considerable, comparisons of blood and biliary concentrations were made after the intravenous injection of penicillin (750 u./kg.) in dogs. Simultaneous measurements were made in saliva collected after cannulation of the submaxillary duct, and augmented by

TABLE 1

Distribution of penicillin in various tissues of rats and rabbits

ANIMAL	CONCENTRATION OF PENICILLIN IN UNITS PER GM OR CC					
	Blood	Brain	Liver	Kidney	Heart	Muscle
Rabbit 1	2	0, 0.15	0.6	80	1.2	0.15
Rabbit 2	2	0, 0	0.6	160	1.2	1.2
Rabbit 3	0.5	0	0	40	0.3	0 3
Rat 1		0	1.4	1 4		
Rat 2		0	4.0	4 5	0	
Rat 3		0	0	8.0	0	0
Rat 4	1 0	0	2.0	7.3	4.4	
Rat 5	2	0	0.9	131.0*	0	1.4
Rat 6	2	0	2.4	11.0	0	0

* On gross inspection this kidney appeared abnormally pale

electrical stimulation of the chorda tympani or the sympathetic nerve supply, and injection of pilocarpine. The results are shown in table 2. The antibiotic activity of saliva before penicillin had been injected was nil by our method, and that of bile was less than the equivalent of 0.03 u. per cc. of penicillin.

Thus, it appears that the amount of penicillin which is lost in the saliva is negligible, while that in the bile may be somewhat greater. However, as shown in the next section, the amount lost in the bile would also appear to have no clinical importance.

Effect of nephrectomy and biliary obstruction. A variable amount of penicillin is quickly excreted in the urine, and this is proportionately larger when the drug is given intravenously than when administered by other routes (6) It has also been shown (1) that the concentration in the blood is maintained at a high level in patients with poor renal function as compared with others. The fate of the penicillin not accounted for in the urine us unknown. It may be destroyed in the tissues or conjugated to an inactive form, lost via the alimentary canal, or destroyed in the kidney.

In an effort to assess the total effect of excretion by the kidneys and destruction in the urine, penicillin (5000 to 10,000 units) was given intravenously to 5 rabbits which had been bilaterally nephrectomized 10 minutes before. In some cases the kidneys were left in situ instead of being removed, after ligatures had been placed tightly around the renal vessels and ureter. The rate of disappearance in the blood then furnished an index of the rapidity of destruction in the body, including that lost into the alimentary canal. Significant amounts of penicillin (0.03 to 0.5 u.) were present in the blood after 24 hours, and even after 48 hours in one instance (0.12 u.), while penicillin could seldom be estimated in the bloods of control animals after 3 hours. Thus, urinary excretion is

TABLE 2

Concentrations of penicillin in blood, saliva and bile of dogs, after intravenous injection

DOG	FLUID	CONCENTRATION OF PENICILLIN IN UNITS PER CC.						
		Minutes after injection of penicillin						
		15	30	45	60	75	90	120
1	Blood	2.0+	1.0	1.0	1.0			
	Bile	1.0	1.0					
	Saliva	0.03	0.06					
2	Blood	1.0						
	Bile	4.0+						
	Saliva							
3	Blood	0.06	0.06	0.06	0.03	0 03	0.03	
	Bile	0.5	4.0+	4.0+	4.0+	4.0+	4.0+	
	Saliva	0	0.5?	0.12	0.5+	0.12	0.5+	
4	Blood		8.0+		4.0		0.5	0.25
	Bile		0.6±		10.0		40+	40+
	Saliva		1.0+		0.25+		0.12+	0.12+
5	Blood		1.0		1.0			
	Bile		5.0+		5.0+			
	Saliva		0 06		0.06			

ordinarily responsible for the major loss of penicillin from the body, although, if this factor is eliminated, the penicillin is otherwise destroyed within a day or two in the body.

The relatively high concentration of penicillin in the bile suggested that a considerable proportion of the agent might be lost in this secretion. To study this factor, the rate of disappearance of penicillin from the blood after intravenous injection was followed in 10 rabbits which had had a ligature placed around the common bile duct from 10 minutes to 2 days before. There was no significant alteration in the curves of blood concentrations from those of the same animals preoperatively. Thus the loss in the bile did not appear to be important.

'ts, both the common bile duct and the kidney were ligated without
tering the effect from that of kidney exclusion alone.

SITE OF DESTRUCTION OF PENICILLIN IN THE BODY. Even though urinary loss of penicillin may be most important clinically, destruction in the tissues must also be considered.

The tissues in which penicillin is most easily destroyed are not known. It is stated (7) that the destruction in shed blood is not rapid, and again (8) that inactivation in serum is considerable. Table 3 shows the comparative rate of destruction in human blood, human urine, and physiological salt solution (U.S.P.) at room, refrigerator and incubator temperatures. When the samples are kept at refrigerator temperature, a delay of several days before analysis is not important, but at room temperature there may be a considerable drop in potency of blood, but not urine, within 2 to 4 days

TABLE 3

Rate of destruction of penicillin in blood, urine and physiological salt solution at refrigerator (4°C.), room (20°C.), and incubator (37°C.) temperatures

		CONCENTRATION OF PENICILLIN IN UNITS PER CC.					
		Original	Days				
			1	2	4	5	7
Blood	4°C.	4		4		4	4
	20°C.	4	4	4	2		0.5
	37°C.	4		0.25		0	0
Urine	4°C	4		4		4	4
	20°C	4		4		4	4
	37°C	4		4		2	1
Physiological salt solution (U S P)	4°C	4		4		4	4
	20°C	4		4		4	2
	37°C	4		1		0 5	0.06

As it appeared possible that the reticuloendothelial cells might destroy penicillin more rapidly than other tissues, an attempt to block these cells by the injection of colloidal material (9) (india ink or trypan blue) was made in 9 rabbits. After subsequent injections of penicillin, the concentrations attained were slightly higher than in previous control runs on the same animals, suggesting that a small, and probably unimportant amount of penicillin is destroyed by reticuloendothelial cells

EVIDENCE AGAINST HEPATIC DESTRUCTION Destruction of penicillin by the liver at a rate more rapid than that by other tissues would impair the efficacy of oral administration, and for this reason might be of clinical importance. To test this possibility, penicillin was injected intravenously into rabbits, and the time-course of the fall in concentration in the blood concentrations determined Then, a few days later, the same amount of penicillin was injected into a branch of the portal vein, and the descent of blood concentration again followed. This experiment was repeated 13 times, using 5000± units of penicillin dissolved in 1 or 2 cc. of physiological salt solution on each occasion. There was no apparent difference in the two curves, suggesting that the liver does not

destroy penicillin more rapidly than other tissues, according to this criterion. The average blood concentrations at 15 minute intervals for 6 different rabbits are shown in table 4.

EFFECT OF PENICILLIN ON TISSUE RESPIRATION. The possible influence of penicillin on tissue respiration in the rat was also investigated. Injection of doses of 1000 units per animal did not modify the oxygen consumption (Warburg method) of cerebral cortex, liver, kidney cortex or diaphragm of animals decapitated 30 minutes after injection. Direct addition of graded doses of penicillin to slices of the same group of organs in respirometer vessels likewise failed to alter the rate of oxygen consumption. In this series the final concentrations of penicillin ranged from 100 to 1400 units per gram wet weight of tissue.

DISCUSSION. The distribution of penicillin in the body, except the tissues of high lipid content, such as the central nervous system, appears to be relatively uniform, and in the general range of that in the blood. The kidneys of some species may contain a concentration much higher than that in the blood, but the element of urinary contamination is not easily avoided.

TABLE 4

Penicillin in blood of rabbits after intravenous injection into ear vein and portal vein
(averages for 6 rabbits)

	CONCENTRATION OF PENICILLIN IN HEART BLOOD			
Time after Injection.	15 minutes	30 minutes	45 minutes	60 minutes
	units per cc	*units per cc*	*units per cc*	*units per cc*
Injection into ear vein	0.46	0.22	0.10	0.06
Injection into portal vein	0.60	0.12	0.10	0.05

The fate of penicillin in the body is only partially accounted for by our results The major part is usually quickly lost in the urine, and only an unimportant fraction in the bile. In the intact animal, immediate passage through the liver does not affect its rate of destruction, but blocking the reticuloendothelial system, of which the liver constitutes a considerable part, may slightly retard its destruction. In any case, the fact remains that destruction of penicillin in the body is of secondary importance to renal excretion. No studies of the destruction of penicillin by organ perfusion were made, or of the possibility that conjugated or otherwise inactivated penicillin might be excreted in the urine.

4. Penicillin is not rapidly destroyed in shed blood at body temperature, although even less rapidly in urine or physiological salt solution.

5. Accordingly, the major portion of any administered penicillin is lost in the urine, with a minor part in bile and saliva, and the fate of the portion unaccounted for remains unknown.

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EFFECT OF VARIOUS ELECTROLYTES UPON THE TOXICITY OF DESOXYCORTICOSTERONE ACETATE

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Previous experiments performed in this laboratory have shown that desoxy-corticosterone acetate (DCA) causes nephrosclerosis, hypertension, periarteritis nodosa and myocardial lesions similar to those seen in acute rheumatic fever (1–4). Under certain conditions, the joints may also be involved and exhibit arthritic and periarthritic lesions resembling those seen in the rheumatic fever of man (32). The relative prominence of the manifestations mentioned above is subject to great variations in different species and even in different individuals of the same species; essentially, however, all animals (fowl, cats, dogs, guinea pigs, rabbits, mice, rats and rhesus monkeys were tested) are sensitive to DCA overdosage inasmuch as they develop some or all of the above-mentioned lesions, when treated with adequate doses over a sufficiently long period (2, 5–8).

It soon became evident that unilaterally nephrectomized rats are particularly sensitive to the toxic manifestations of DCA, especially if they are maintained on a high sodium chloride intake (9). Female or castrate male rats, are more sensitive than intact males and administration of testoid compounds, such as methyl-testosterone, raises resistance against DCA (10, 11). Ammonium chloride, which is an "acidifying salt," is extremely potent in antagonizing the toxic manifestations of DCA overdosage (12), without significantly influencing the life-maintaining action of this steroid, when tested on the adrenalectomized rat (13).

These observations promise to have certain practical applications. If in man, as in animals, ammonium chloride should prove to prevent the toxic actions of DCA without interfering with its life-maintaining potency, the corticoid compound could be administered to patients with greater safety if they simultaneously received ammonium chloride. Indeed, the practical applications of this experimental work may not limit themselves solely to patients receiving DCA medication. There is reason to believe that excessive endogenous production of salt-active corticoids by the adrenal cortex may be responsible for the production of renal hypertension, periarteritis nodosa and even certain types of myocarditis which occur spontaneously in man (14). It has been shown that crude preparations of cattle anterior pituitary tissue share with DCA the ability to elicit nephrosclerosis, periarteritis nodosa and myocardial nodules (15). Since these same preparations also cause marked adrenal cortical enlargement, it is perhaps not unreasonable to assume that their toxic manifestations are mediated by the adrenal cortex, which under their influence produces excessive amounts of salt-active corticoids. That there is a close

relationship between the toxic manifestations elicited by DCA and those produced by anterior lobe preparations is made even more probable by the recent observation that ammonium chloride prevents not only the toxic effects of DCA, but also those of the anterior lobe preparations (16).

Assuming that our tentative interpretation is correct, the most important task will be to determine what pathogenic stimuli can increase the endogenous production of DCA-like corticoid compounds. It is well to remember in this connection that adrenal cortical hyperplasia and hypertrophy, accompanied by signs of increased corticoid hormone production, represent an integral part of the so-called "Alarm Reaction" and indeed of the entire "General Adaptation Syndrome" (17). In accordance with expectations, it was possible to demonstrate that various damaging agents which cause adrenal cortical enlargement, also produce organ lesions essentially similar to those elicited by DCA or anterior lobe extract. Thus, for instance, exposure to cold, if continued for a period of several weeks, causes nephrosclerosis and hypertension. In this case again, unilateral nephrectomy and administration of NaCl proved to act as sensitizing agents (18).

The above-mentioned observations are most readily compatible with the concept that those diseases of man, which are simulated by DCA over-dosage, are actually "diseases of adaptation." Non-specific damaging agents (infections, allergic reactions, stress and strain) stimulate—for the purpose of defense and adaptation—the adrenotropic hormone production of the anterior lobe. The latter augments the production of DCA-like corticoid compounds by the adrenal cortex and these in turn (perhaps through their effect on electrolyte metabolism) produce the typical organ lesions.

There is convincing experimental evidence showing that thyroxin likewise sensitizes animals to the toxic effects of DCA and hence it is not improbable that in human pathology an excessive production of thyrotropic hormones may also play an important, though subsidiary, rôle in the pathogenesis of the so-called "diseases of adaptation" (19).

If this interpretation is correct, ammonium chloride, which counteracts the toxicity of DCA, may prove of therapeutic value even in diseases resulting from endogenous over-production of corticoid hormones. It is mainly for this reason that we undertook the present experimental series in which we examined the action of a number of electrolytes upon the toxic manifestations of DCA in the rat. When we began these experiments, we already knew that sodium chloride aggravates, while ammonium chloride inhibits the development of DCA over-dosage phenomena. The question arose whether the sensitizing and inhibitory actions, respectively, are due to the salts as a whole, to specific pharmacologic actions of the anion or cation, or to secondary changes in the alkali reserve and pH of the body fluids, which might result from the administration of such salts.

EXPERIMENTAL. 17 groups, each consisting of 12 unilaterally nephrectomized female albino rats weighing 130–160 g. (average 139 g.) were used for the experiments to be reported in this communication. At the time of operation all animals, except those in Groups 1 and 4, received subcutaneously, a pellet of DCA compressed at a pressure of 1,000 lbs.

per cm². The rather complex experimental arrangement is briefly outlined in the column headed "Treatment" of table 1. All animals received tap water to drink and "Purina Fox Chow"[1] to eat (both *ad lib*), and all solutions indicated were administered daily by gavage. Groups 1, 2, 4 and 6 may be regarded as various types of controls to be used for comparison with the other groups. Group 1 merely received water by stomach tube, without any hormone treatment. Group 2 received water and DCA, hence indicating the degree of toxicity exhibited by DCA without additional electrolyte treatment. Group 4 received a 6% NaCl solution daily by gavage and served as a control for Groups 6, 8–17, all of which received NaCl, in the same concentration and amount, as Group 4. In addition to this NaCl treatment, Group 6 received a DCA pellet and hence served as a control, for all groups receiving DCA and the "Standard Concentration" (6%) of NaCl. Group 3 received DCA and Na₂SO₄, the latter in a concentration containing the same number of m. eq. as the 6% NaCl solution in Group 4. The purpose of this group was to determine whether Na given in the form of a salt other than NaCl, would be equally effective in sensitizing the animals to the toxic actions of DCA. Groups 5 and 7 were included to determine the relationship between the concentration of the NaCl administered and its ability to increase the toxicity of DCA. In Group 5 a quarter of the "Standard Concentration" and in Group 7, twice the "Standard Concentration" were administered. The electrolytes which were given in addition to NaCl in Groups 8, 9, 10, 11, 14 and 15 were introduced in concentrations containing the same number of m. eq. as the "Standard" NaCl solution. Thus in Group 8 the animals received NaCl and NH₄Cl in concentrations whose chloride content was equivalent. This was done in order to compare the protective effect of NH₄Cl with that of other salts of equivalent anion concentration. The concentration of the di- and monobasic phosphates (Groups 12 and 13) was similarly calculated, on the assumption that their bulk would dissociate to give HPO₄⁻ and H₂PO₄⁻ respectively.

As previously stated some of the organ changes produced by excessive amounts of DCA resemble those of human rheumatic fever, hence it appeared of interest to establish whether salicylates—so active in combatting the manifestations of the human disease—would have a protective action against the experimentally produced lesions. Because of the toxic action of sodium in DCA-treated animals, it appeared advisable to use ammonium salicylate rather than sodium salicylate for this work. Since salicylates are not tolerated in concentrations corresponding to the "Standard" NaCl solution, the ammonium salicylate in Group 16 was given in the form of a 1% solution. No gavages were given on the day of operation or the following day, in order that the animals might recover. After this, the same amount of fluid was given to all animals irrespective of the concentration and number of salts administered. On the second day after the operation, all animals received one gavage of 2 cc. and on all subsequent days during the first three weeks of the experiment they received two daily gavages of 2 cc. After three weeks this was increased to 3 cc. twice daily. This sequence was somewhat interrupted on days when blood samples were taken for analysis, as will be explained later.

The animals were sacrificed after six weeks and the organs were fixed in "Suza" mixture, weighed and histologically examined. The degree of nephrosclerosis and the extent of myocardial damage were estimated in each animal and expressed in a scale ranging from "0" to "+ + +." The percentual intensity of nephrosclerosis and cardiac lesions was computed by adding the total number of plus signs in each group and then dividing by the number of animals in the group. The resulting figure was then expressed as a percentage of the theoretical maximum of "+ + +" in all animals of a group. This procedure gives a fair estimate of the average intensity of the lesions. The average incidence was similarly computed disregarding the intensity of the change and taking every organ with a demonstrable lesion as positive. The incidence of periarteritis nodosa, as detected in the mesenteric vessels, was also computed in this manner. The weight of the heart (ventricles only) and of the kidney was expressed as a percentage of the final body weight; the standard errors

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[1] Ralston Purina, Woodstock, Ontario.

of these figures are also listed. The water intake was measured daily and the averages are shown in the table for the two weeks preceding the day mentioned.

The average pellet weight at the time of implantation was 44.6 mg. and during the six weeks of the experiment an average of 11.8 mg. was absorbed. It is to be noted that the rate of absorption was essentially the same in all groups.

It would appear from the final body weight and low water intake of Group 5 (see table 1) that this group was damaged by the treatment. However, the animals appeared healthy in all respects and the body weight of the groups receiving higher concentrations of NaCl (Groups 4, 6, and 7) were not below average.

Though only three animals survived in Group 3, three others died during the last three days of the experiment and were included in our averages. All other animals (in any group) which died from incidental causes before the sixth week were not included in the averages.

Samples of blood were taken on the 14th, 28th and 42nd day of treatment after the animals had been fasted for 16 hours. The last gavage was given to the first half of each group of rats 16 hours, and to the second half, 1 hour previously. On the 14th and 28th days, the blood was taken from the jugular vein while the animals were under light ether anesthesia. It was collected under oil, allowed to clot, then centrifuged and aliquots measured out as soon as possible. The final blood sample, taken on the 42nd day, was obtained by cutting the jugular vein and exsanguinating the rat as completely as possible. Part of the sample was put in an oxalated bottle, while the rest was placed in a centrifuge tube, covered with oil and allowed to clot. The second daily gavage normally given, was omitted on the 14th and 28th days.

Chloride determinations were done using a micro-modification of Van Slyke's (20) method; sodium by a modification of the method of McCance and Shipp (21) suggested by A. 'Neufeld (private communication); potassium by Jacobs and Hoffman's (22) procedure; calcium by that of Clark and Collip (23) and finally phosphorus by a photo-electric modification of the method of Fiske and Subbarow (24). The results are expressed in m. eq. per litre of blood or serum.

As regards the morphologic manifestations of DCA overdosage, perusal of table 1 indicates that under the conditions of these experiments, in animals receiving no additional electrolyte treatment (Group 2), a single subcutaneously implanted pellet of DCA caused only mild nephrosclerosis; cardiac nodules were entirely absent and the incidence of periarteritis nodosa was low. Correspondingly, the increase in the percentual weight of the kidney and heart was not very pronounced. If the values of this group are compared with those of the group receiving the "Standard" amount of NaCl in addition to DCA (Group 6), it is immediately evident that, in agreement with our previous observations, NaCl greatly sensitizes the experimental animals to the toxic manifestations of the corticoid hormone. Nephrosclerosis and cardiac lesions were present in almost every animal and their intensity was severe. Periarteritis nodosa of the mesenteric vessels was likewise of common occurrence and correspondingly the weights of kidney and heart were far above normal. These toxic manifestations of DCA and NaCl on the kidney, were even more pronounced in the group receiving twice the "Standard" concentration of NaCl (Group 7), but one-quarter of this amount of NaCl (Group 5) led to lesions which were only very slightly more severe than those caused by the same amount of DCA in animals given tap water only (Group 2). These observations show that the minimum effective dose of NaCl is approximately that given in Group 5. It appears furthermore, that our "Standard" amount of NaCl is probably not

TABLE 1

Summary of organ changes and biochemical findings in the first experimental series

GROUP NO.	TREATMENT	FINAL BODY WEIGHT gms.	NEPHRO-SCLEROSIS % Incidence	NEPHRO-SCLEROSIS % Intensity	CARDIAC LESIONS % Incidence	CARDIAC LESIONS % Intensity	PERIARTERITIS NODOSA % INCIDENCE	ORGAN WEIGHTS AS % OF BODY WEIGHT Kidney	ORGAN WEIGHTS AS % OF BODY WEIGHT Heart	DAY OF EXPERIMENT	NO OF ANIMALS 16*	NO OF ANIMALS 1*	DAILY WATER INTAKE cc/rat	WHOLE BLOOD Cl 16*	WHOLE BLOOD Cl 1*	SERUM Cl 16*	SERUM Cl 1*	SERUM Na 16*	SERUM Na 1*	SERUM Na/Cl 16*	SERUM Na/Cl 1*	SERUM K 16*	SERUM K 1*
1	H₂O	103 ±4 1	0	0	5	0	0	0.52 ±0 01	0.34 ±0 01	14	6	6	18	71 ±1 4	74 ±1.2	00 ±1.7	92 ±1.7	141 ±2.5	138 ±1 8	1.58 ±0.03	1.46 ±0.04	4.78 ±0.22	3.97 ±0.10
										28	8	8	22			92 ±1.1	92 ±0.5	143 ±2.3	137 ±2.4	1.55 ±0.03	1.48 ±0.03	4.07 ±0.28	3.75 ±0.11
										42	0	0	22			09 ±0.7	07 ±1.3	151 ±2.3	152 ±1.5	1 51 ±0 03	1.56 ±0.03	5.05 ±0.28	5.17 ±0.17
2	DCA + H₂O	151 ±3.3	0	0	5	0	8	0.60 ±0 01	0.40 ±0.01	14	5	0	21	84 ±2 7	64 ±2.1	84 ±0.7	83 ±1.0	144 ±3.5	137 ±8.0	1.69 ±0.03	1.55 ±0.07	3.50 ±0.25	3.07 ±0.29
										28	5	0	28			80 ±0.8	85 ±1.6	140 ±3 0	141 ±1.2	1.62 ±0.03	1.85 ±0.03	2.29 ±0.19	2.33 ±0.43
										42	5	0	28			92 ±1.0	89 ±1.4	153 ±2.4	151 ±3.6	1.66 ±0.05	1.69 ±0.04	3.00 ±0.13	3.21 ±0.47
3	DCA + Na₂SO₄	170 ±4 2	33	20	33	11	0	0 77 ±0 51	0.45 ±0 02	14	5	8	31	49 ±1 2		61 ±3 7	70 ±1.0	151 ±1.8	155 ±1.8	2.53 ±0.21	2.22 ±0.03	1.37 ±0.15	2.22 ±0.39
										28	5	0	37			71 ±1.5	74 ±3.4	141 ±0.5	157 ±2.8	1.98 ±0.09	2.12 ±0.0	1.70 ±0.28	2.02 ±0.42
										42	3		31			66 ±1.7		181 ±3.5		2.40 ±0.09		1.29 ±0.15	
4	NaCl	199 ±4.5	4	1	0	0	0	0 58 ±0 02	5 34 ±0.01	14	5	5	28	73 ±2 7	85 ±2.1	87 ±2 4	87 ±1.4	143 ±3.3	139 ±2.8	1.64 ±0.00	1.60 ±0.05	5.13 ±0.15	3.88 ±0.18
										28	5	0	35			87 ±0.7	100 ±1.2	139 ±2.5	154 ±3.4	1.58 ±0.04	1.53 ±0.03	4.09 ±0.20	3.47 ±0.23
										42	5	5	40			98 ±2 2	110 ±1.7	144 ±2.0	150 ±3.8	1.47 ±0.04	1.48 ±0.0	5.31 ±0.31	5.07 ±0.40
5	DCA + ½ NaCl	157 ±8 9	18	10	18	9	18	0 68 ±0 03	0.39 ±0.01	14	5	5	18	52 ±0 9	51 ±2.4	85 ±1 1	92 ±0.5	141 ±3.1	144 ±3.4	1.64 ±0.04	1.56 ±0.05	2.65 ±0.32	2.43 ±0.22
										28	5	0	19			82 ±2.4	83 ±3.3	134 ±3.5	129 ±2.5	1.59 ±0.03	1.52 ±0.03	2.00 ±0.20	1.49 ±0.19
										42	5	3	19			85 ±1.8	135	135 ±3.5	141 ±2.0	1.55 ±0.07	1.43 ±0.01	3.58 ±0.15	3.05 ±0.19
6	DCA + NaCl	184 ±3.5	54	75	100	55	65	0.78 ±0.02	0 48 ±0.01	14	0	5	39	63 ±3.0	77 ±5.7	78 ±1.4	80 ±1 8	151 ±3.5	148 ±2.4	1.01 ±0.08	1.84 ±0.06	3.72 ±0.35	2.00 ±0.31
										28	0	0	51			75 ±1.2	02 ±1.7	143 ±2.5	155 ±3.3	1.89 ±0.03	1.69 ±0.03	1.62 ±0.21	1.95 ±0.32
										42	4	3	51			89 ±1.0	102 ±2.3	155 ±1.8	164 ±0.7	1.72 ±0.03	1.60 ±0.04	3.73 ±0 38	3.56 ±0.23
7	DCA + NaCl + NaCl	171 ±4.8	100	77	83	39	65	0.93 ±0 10	5.45 ±0 03	14	8	0	57	59 ±3.2	71 ±3.5	76 ±1.6	89 ±1.0	144 ±1.2	152 ±2.7	1.87 ±0.03	1.82 ±0.03	2.95 ±0.30	2.59 ±0.08
										28	3	4	75			88 ±3.0	104 ±7.9	137 ±1.0	151 ±2.4	1.54 ±0.04	1.40 ±0.10	2.27 ±0.37	3.07 ±0.25
										42	3	3	79			84 ±4.0	103 ±2.0	152 ±0.4	197 ±2.4	1.82 ±0.00	1.63 ±0.00	2.85 ±0.81	3.83 ±0.60

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	Treatment																				
8	DCA + NaCl + 195 NH4Cl ±4 6																				
9	DCA + NaCl + 189 CaCl2 ±3 4																				
10	DCA + NaCl + 193 (NH4)2SO4 ±3 0																				
11	DCA + NaCl + 193 NH4NO3 ±3 0																				
12	DCA + NaCl + 187 (NH4)2HPO4 ±3 2																				
13	DCA + NaCl + 190 NH4H2PO4 ±5 0																				
14	DCA + NaCl + 193 MgCl2 ±4 6																				
15	DCA + NaCl + 189 KCl ±1 6																				
16	DCA + NaCl + 191 NH4 salicylate ±8 0																				
17	DCA + NaCl + 187 K acetate ±5 2																				

*Time in hours after gavage

far from the maximally effective dose, since even twice the "Standard" amount (Group 7) does not cause much more severe lesions.

Na_2SO_4 (Group 3) shares with NaCl the ability to increase the toxicity of DCA, yet in the present series it appears to have been quantitatively less effective in producing morphologic manifestations of overdosage. It will be noted, however, that this electrolyte caused very severe blood chemical changes and proved particularly damaging to our animals (high mortality).

NH_4Cl (Group 8) was highly potent in counteracting the toxic effects of DCA given in combination with NaCl. This beneficial effect manifested itself in the complete prevention of periarteritis nodosa and a marked decrease in the incidence and intensity of both nephrosclerotic and cardiac lesions. The increase in kidney weight occasioned by DCA (especially when given in combination with NaCl) was not inhibited by NH_4Cl; on the other hand, the cardiac enlargement which is probably secondary to the hypertension caused by the nephrosclerosis, was significantly inhibited by the NH_4Cl. All these observations confirm our earlier findings concerning the actions of NH_4Cl (12).

Calcium chloride (Group 9), ammonium sulphate (Group 10) and, to a lesser degree, ammonium nitrate (Group 11) shared the beneficial effect of ammonium chloride. This suggests that neither the cation nor the anion of ammonium chloride is indispensable for this action. On the other hand, all salts with anti-nephrosclerotic potency (Groups 8, 9, 10 and 11) have in common the ability to act as "acidifying agents", that is, to depress the alkali reserve of the blood; hence it is suggested that perhaps the beneficial effect, of the electrolytes studied, is not due to the chemical identity of either cation or anion, but is connected in some manner with their ability to produce, within the organism, an excess of acidifying anions. In the case of ammonium chloride, ammonium sulphate and ammonium nitrate, this is probably achieved because the NH_4^+ ion is neutralized by being transformed into urea, thus leaving an excess of acid; in the case of calcium chloride the comparatively low absorption rate of calcium and its ready excretion into the intestine achieves the same end result, leaving an excess of Cl ions to form HCl in the body. Further experiments will have to show whether the DCA-antagonizing effect of such an anion excess is due to their combination with sodium, which helps to deplete the body of the latter or whether they act directly.

The di- and monobasic phosphates (Groups 12 and 13) proved to be devoid of any noteworthy anti-nephrosclerotic action. It does not appear profitable, however, to discuss the reasons for this apparent inactivity because histologic examination of the kidneys showed that in these two groups calcified cylinders were formed throughout the kidney, within the distal convoluted tubules and collecting tubules, presumably because of the high phosphate concentration of the urine. By obliterating the lumina of the nephrons, these cylinders led to considerable destruction of renal tissue and hence it was difficult to determine how much of the kidney damage was secondary to this and how much was due to the toxicity of DCA. It may be remarked parenthetically that unfortunately these tissues were fixed in Suza solution and not in neutral formalin,

so that the histochemical identification of the calcium by Kossa's stain was not feasible. The general histological characteristics of the cyclinders, including their purple coloration with hematoxylin was quite characteristic, however, and in view of the fact that high phosphate intake is known to cause calcium precipitation within the nephrons, it appears reasonably safe to assume that the cylinders were calcified. Be this as it may, the phosphates proved useless in antagonizing the action of DCA.

Magnesium chloride (Group 14) and potassium chloride (Group 15), likewise failed to effect any significant decrease in the toxic actions of DCA and as regards KCl this inactivity was even more clearly shown in a later experimental series (table 2). This is noteworthy since some investigators believed that all toxic manifestations of DCA, and particularly its damaging effect upon renal and cardiac structure, are secondary to its blood potassium depressing effect. Indeed, it has been stated by some that the administration of KCl antagonizes the actions of DCA in the rat (25–27). We cannot confirm this under the conditions prevailing in our experiments and, since KCl is not an acidifying salt, no beneficial effect may be expected of it on the basis of the hypothesis expressed above. In the case of magnesium chloride, the inefficacy of our treatment may have an entirely different explanation, inasmuch as the animals of this group developed a very pronounced diarrhea under the influence of the orally administered electrolyte, so that probably a large portion of it went through the intestinal tract unabsorbed. The salts of organic anions, namely the salicylate (Group 16) and the acetate (Group 17) were likewise ineffective. In the case of the salicylate, it will be remembered that the concentration was not comparable with that of the other electrolytes and in the case of the acetate, we may assume that the organic anion was metabolized. It will be seen later (table 2) that another organic anion, namely citrate, is likewise ineffective even if given in the form of ammonium citrate, in which the cation is probably neutralized within the body as a result of urea formation.

It is noteworthy, however, that the incidence and intensity of the cardiac and periarteritic lesions was more or less significantly lower in Groups 8–17 than in the control group receiving DCA with NaCl alone, that is, without any additional electrolytes (Group 6).

The water intake and consequently also the diuresis was slightly above normal in the animals receiving DCA (Group 2), considerably above normal in those receiving DCA with the "Standard" amount of NaCl (Group 6) and highest in the group receiving DCA and twice the "Standard" amount of NaCl (Group 7). Perusal of Groups 8–17 indicates that under the conditions of our experiments, sodium chloride raised the water intake more effectively (Group 7) than equivalent concentrations of electrolyte mixtures in which only half of the solute is represented by sodium chloride (Groups 8–17).

In confirmation of our previous observations (28–30), we note that DCA tends to depress both the whole blood and the serum chlorides. This effect was particularly pronounced in the group receiving DCA and sodium sulphate (Group 3). Indeed, in this group the hypochloremia was so severe, that it in

itself may perhaps explain the high mortality rate observed in this group. It is interesting to note that exogenous administration of NaCl, far from compensating for this hypochloremia, actually increased it in the DCA-treated animals, except immediately after the gavage. (Compare Group 2 with Groups 6 and 7).

Although the Na/Cl ratio is rather variable, we note in agreement with earlier findings that this index is highest in the animals most severely damaged by DCA, that is the sodium sulphate group (Group 3) in which the mortality rate was highest and the two groups receiving comparatively high concentrations of NaCl without any additional electrolytes (Groups 6–7).

It is noteworthy that both overdosage with a corticoid compound and adrenalectomy cause a similar depression of the blood chloride level. This has its clinical equivalent inasmuch as it has been observed that the blood chlorides tend to be subnormal in Cushing's disease as well as in Addison's disease (31). There are striking similarities between Cushing's disease and experimental DCA overdosage; in both these conditions the blood pressure tends to be raised, there is cardiac hypertrophy, a rise in the blood volume and frequently nephrosclerosis. We found, furthermore, that in NaCl-treated rats, anterior lobe extracts cause the same morphologic manifestations of hormone overdosage as does DCA (15) and that the lesions normally produced by anterior lobe extracts are also preventable by ammonium chloride treatment (16). Hypopotassemia, such as is occasioned by DCA overdosage, can also occur in patients with Cushing's disease (31) and has been observed under certain conditions in our laboratory in rats treated with anterior pituitary extracts (unpublished data). All these observations imply a close relationship between Cushing's disease and the experimental lesions induced by overdosage with anterior lobe extracts or DCA. It is questionable, however, whether the pituitary influences the blood chlorides solely through the intermediary of the adrenocorticotropic hormones; posterior lobe extracts also affect chloride metabolism and, unlike in the intact rat, DCA does not cause hypochloremia in the hypophysectomized animal (29).

The decrease in serum potassium, which usually follows DCA overdosage, is obvious in Group 2 and most severe in the group receiving DCA in combination with sodium sulphate (Group 3). Perusal of the other figures indicates, however, that there is no close relationship between the blood potassium level and the damaging effect of the corticoid hormone. Thus administration of ammonium sulphate (Group 10), ammonium chloride (Group 8), calcium chloride (Group 9) or ammonium nitrate (Group 11) failed to restore the abnormally low blood potassium of the DCA treated animals to normal, yet all of these electrolytes were highly effective in counteracting the toxic manifestations of the hormone, as judged by the scarcity of organ changes. Conversely, KCl (Group 15) which raised the blood potassium far above normal one hour after gavage, and maintained it at approximately normal levels even 16 hours after gavage, failed to inhibit significantly the nephrosclerotic effect of DCA.

The most outstanding facts which emerge from this first experimental series are that sodium given either as a sulphate or as a chloride sensitizes the rat to the toxicity of DCA, while certain acidifying salts tend to protect it. Potas-

sium cannot take the place of sodium as a sensitizing agent, nor does it have any noteworthy protecting effect against DCA. Salts with organic anions likewise fail to give any marked protection against the toxicity of the corticoid compound.

In order to obtain further data in connection with these main conclusions, we performed a second experimental series under slightly different conditions.

In this series we used 13 groups, each consisting of 10 female albino rats weighing from 40–60 g. (average 50 g.). Except for $NaHCO_3$, the compounds used were investigated both alone and simultaneously with NaCl. It will be noted that some of the electrolytes examined were not included in the previous series.

Since these animals were smaller, a lower "Standard" NaCl concentration, 2.5%, was used. However, this was increased on the 10th day to 3.5% and on the 20th day to 4.5%. The amount given was always 2 cc. twice daily. The animals were sacrificed after four weeks as we wished to determine to what extent the lesions would be manifest at the end of this shorter period. Blood chemical determinations were performed only on the last day of the experiment 16 hours after gavage. All other details were essentially the same as in the first series.

Perusal of table 2 indicates that, although the experimental arrangement was slightly different in this series, the results were essentially the same as those of the first experiment. Probably because of the shorter duration of treatment, however, neither the heart weights nor the histological lesions in the heart were sufficiently pronounced, to deserve a special discussion. The incidence and intensity of nephrosclerosis in the groups receiving no NaCl supplements was comparatively low in all groups (Groups 2–6). It was lowest in those receiving NH_4Cl (Group 3) or $CaCl_2$ (Group 4), thus confirming the antinephrosclerotic potency of these electrolytes. On the other hand, neither KCl (Group 5) nor ammonium citrate (Group 6) showed any nephrosclerosis-inhibiting potency in comparison with the DCA-treated controls (Group 2).

$NaHCO_3$ (Group 7) proved approximately as potent as NaCl (Group 8) in sensitizing the animals to this toxic effect of DCA.

Among the animals which were treated simultaneously with DCA and the "Standard" NaCl solution (Groups 8–13) the nephrosclerosis was consistently pronounced except in the groups receiving either NH_4Cl (Group 10) or $CaCl_2$ (Group 11). In this series KCl and ammonium citrate were again ineffective, thus furnishing additional support in favor of the concept that only acidifying salts possess a beneficial action.

It will also be seen from table 2, that the increase in the percentual weight of the kidney caused by DCA (and to an even greater extent by DCA in combination with various electrolytes) is not inhibited even by those salts which are highly potent in counteracting the nephrosclerotic effect of the corticoid compound. The interpretation of this "renotropic" action of DCA is rather difficult on the basis of data available to us at the present time. In the past, that is before we learned how to inhibit the nephrosclerotic action of DCA, it

TABLE 2

Summary of organ changes and biochemical findings in the second experimental series

GROUP NO.	TREATMENT	FINAL BODY WEIGHT	NEPHRO-SCLEROSIS		KIDNEY WEIGHTS AS % BODY WEIGHT	NO OF ANIMALS	DAILY WATER INTAKE	WHOLE BLOOD Cl	SERUM			
			% Incidence	% Intensity					Cl	Na	Na/Cl	K
		grams					cc/rat					
1	H₂O	94 ±5.3	0	0	0.67 ±0.03	6	12	70 ±0.6	87 ±1.1	142 ±3.1	1.63 ±0.05	5.40 ±0.19
2	DCA + H₂O	103 ±4.3	25	8	0.76 ±0.02	10	18	60 ±1.1	77 ±1.2	115 ±1.8	1.88 ±0.03	3.28 ±0.24
3	DCA + NH₄Cl	114 ±4.2	15	5	0.84 ±0.03	10	23	64 ±1.0	81 ±1.0	140 ±1.5	1.68 ±0.03	3.47 ±0.31
4	DCA + CaCl₂	87 ±7.1	8	3	0.82 ±0.05	6	17	63 ±1.4	90 ±1.6	139 ±5.2	1.55 ±0.08	6.15 ±0.55
5	DCA + KCl	100 ±4.4	43	25	0.82 ±0.01	8	18	60 ±2.1	87 ±0.8	153 ±3.3	1.78 ±0.04	5.06 ±0.23
6	DCA + (NH₄)₂-citrate	112 ±6.1	56	27	0.83 ±0.03	8	18	53 ±0.8	83 ±1.4	137 ±3.5	1.65 ±0.05	3.12 ±0.23
7	DCA + NaHCO₃	101 ±3.4	77	35	0.93 ±0.02	9	23	48 ±1.1	72 ±0.9	143 ±1.6	2.02 ±0.03	2.94 ±0.36
8	DCA + NaCl	112 ±3.7	90	61	0.81 ±0.03	10	24	60 ±3.4	78 ±1.4	144 ±1.8	1.81 ±0.04	2.76 ±0.27
9	DCA + NaCl + NaCl	91 ±5.3	82	55	0.96 ±0.03	9	30	57 ±1.6	88 ±0.5	151 ±1.8	1.71 ±0.02	4.16 ±0.21
10	DCA + NaCl + NH₄Cl	108 ±3.6	5	1	0.91 ±0.02	10	26	60 ±1.9	84 ±2.1	153 ±3.2	1.84 ±0.04	3.83 ±0.35
11	DCA + NaCl + CaCl₂	97 ±7.0	40	12	0.90 ±0.01	5	21	50 ±1.1	84 ±3.1	154 ±0.9	1.83 ±0.06	4.46 ±0.40
12	DCA + NaCl + KCl	109 ±5.1	82	51	0.92 ±0.03	9	28	63 ±1.1	86 ±1.1	156 ±2.0	1.82 ±0.05	5.07 ±0.25
13	DCA + NaCl + (NH₄)₂-citrate	101 ±5.1	72	15	0.93 ±0.02	8	25	54 ±0.8	79 ±2.4	147 ±2.9	1.87 ±0.08	3.94 ±0.52

was impossible to detect any renotropic action in this steroid since the kidney-stimulating effect, if any, was masked by the pathologic changes. A study of the histologic slides, prepared from the kidneys of the animals described in this communication, clearly indicates however that the epithelial cells of the proximal and distal convoluted tubules are hypertrophic and the tubular lumina tend to be distended in animals in which the nephrosclerotic action of DCA was counteracted by suitable electrolyte treatment.

The blood chemical studies yielded rather variable results in this experiment. They confirm, however, that DCA tends to lower the serum chlorides and thus raises the serum Na/Cl ratio, while the blood potassium is decreased; yet there is no direct relationship between the degree of hypochloremia or hypopotassemia and the intensity of the resulting nephrosclerosis. Thus in the group receiving twice the "Standard" amount of NaCl (Group 9) the Na/Cl ratio was lower than in the group receiving NaCl in combination with NH$_4$Cl (Group 10) although in the former, the nephrosclerosis was very pronounced while in the latter it was usually absent. Similarly, KCl raised the blood potassium to or above normal (Groups 5 and 12) without preventing nephrosclerosis. These observations led us to conclude that a high Na/Cl ratio and a low potassium are only approximate indices of the degree of DCA intoxication, but are not the direct cause of its nephrosclerosis-producing activity. Experiments now under way in this Laboratory (especially those concerned with blood pH, alkali reserve and tissue electrolyte concentration) may perhaps give us more accurate information concerning the pathogenetic mechanism responsible for the cardiovascular lesions.

SUMMARY. The experiments described in this communication confirm that unilateral nephrectomy and large amounts of sodium chloride sensitize the rat to certain morphologic manifestations of DCA overdosage (nephrosclerosis, cardiac lesions and periarteritis nodosa).

The sensitization by NaCl is not due to the Cl ion, but to the Na ion, as indicated by the fact that other chlorides are ineffective in this respect, while other Na salts share this action of NaCl.

A number of electrolytes were tested for their ability to prevent the renal and cardiovascular damage normally elicited by DCA. Among these, only the "acidifying" salts (ammonium sulphate, ammonium chloride, ammonium nitrate and calcium chloride) proved to possess any noteworthy protecting activity.

Although we confirm that DCA causes a decrease in the chloride and potassium content of the serum, neither of these changes can be made directly responsible for the morphologic manifestations of DCA overdosage. Treatment with electrolytes which restore to normal the serum chloride or serum potassium values, does not necessarily prevent the cardiovascular lesions produced by DCA in the rat.

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THE BLOCKING ACTION OF THE CINCHONA ALKALOIDS AND CERTAIN RELATED COMPOUNDS ON THE CARDIO-INHIBITORY VAGUS ENDINGS OF THE DOG[1]

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INTRODUCTION. A knowledge of the plasma concentration at which a drug exerts its effects and of the procedure by which such concentrations may be maintained is essential to the controlled therapeutic use of the drug. The recent development of good analytical methods has made it possible to study the cinchona alkaloids in this respect. Although much attention has been focussed on their effective plasma concentration as antimalarials, there are other important effects of these drugs which have not been adequately investigated.

It is known that the cinchona alkaloids and certain of their derivatives have a blocking action at neuromuscular junctions, autonomic (15, 16) as well as skeletal (3, 7, 12, 13). But in none of the experiments on this subject has the effective plasma concentration been measured. It was the purpose of this investigation to determine the plasma concentrations at which the cinchona alkaloids (quinine, quinidine, cinchonine, cinchonidine) and certain related compounds block the cardio-inhibitory action of the vagus nerves on the heart.

A. *Experimental:* Dogs were lightly anesthetized by a slow intravenous injection of pentobarbital sodium, or were decerebrated under ether anesthesia. The right vagus nerve was exposed, sectioned high in the neck and the central end of the peripheral part of the nerve laid over an electrode shielded in glass tubing. This electrode was connected to a stimulator arranged to give a one-second tetanic stimulus at the rate of 150 shocks per second. The right carotid artery was cannulated in order to record arterial blood pressure with a mercury manometer and a kymograph. Electrodes placed on the fore limbs were connected to an electrocardiograph. An infusion set-up was arranged to deliver a saline solution of one of the drugs into a hind leg vein at a constant rate. The alkaloids were administered in the form of sulfates except where otherwise indicated.

After determining the voltage necessary to give a maximal cardio-inhibitory response, control recordings of the effect of peripheral stimulation of the right vagus nerve were taken with the kymograph and the electrocardiograph. The infusion was then started and at intervals thereafter records of the effect of vagus stimulation and blood samples were taken. When it was observed that a maximal stimulation of the nerve produced no decrease in blood pressure or slowing of the heart beat, the infusion was stopped to allow the concentration of the drug in the body fluids to fall until the effect of vagus stimulation returned. (Occasionally it was necessary to supplement the infusion solution with intravenous injections in order to obtain a complete vagus block.) This procedure could often be carried through twice in one experiment. In some experiments both vagus nerves were sectioned and tested in the manner described above.

B. *Chemical Methods:* The plasma concentrations of the cinchona alkaloids, n-butyl apocupreine, ethyl dihydrocupreine, hydroxyethylapocupreine, and ethylapocupreine were

determined by the method of Brodie and Udenfriend (1). Quinine methochloride and quinine ethochloride concentrations were determined fluorometrically on plasma filtrates obtained after precipitation of proteins with metaphosphoric acid in the manner described by Brodie and Udenfriend (2) for quinine estimation in plasma. In order to analyse the quaternary nitrogen salt p-hydroxy-acetaminophenyl-hydroxy-ethyl-apocupreinium chloride which is not fluorescent and insoluble in ethylene dichloride, it was necessary to use a modification of the method of Brodie and Udenfriend (1), allowing the compound to react with methyl orange first in aqueous solution and then extracting the combination product in ethylene dichloride for colorimetric estimation.

TABLE 1

Plasma concentration required to block the cardiac effects of peripherol vagus stimulotion

The compounds are listed in order of decreasing effectiveness

DRUG	PARTIAL BLOCK	COMPLETE BLOCK
	mgm. per liter	*mgm. per liter*
Quinidine...	5.0	7.4
n-butyl apocupreine (M.I. No. 76)*.........	5.0	7.6
Quinine ethochloride†...................	6.9	9.3
p-hydroxyacetaminophenyl-hydroxyethylapocupreinium		
HCl Chloride (M.I. No. 102)*.....................	5.0	13.0
Cinchonine...	6.0	13.3
Cinchonidine.........	6.5	15.5
Ethyl dihydrocuprein (M.I. No. 0)*..	8.6	
Quinine methochloride†..	10.0	20
Quinine...........	12.1	19.8
Hydroxyethylapocupreine (M.I. No. 72)*	(no effect up to 14.9)	

* These compounds were obtained through the courtesy of R. S. Tipton of the Mellon Institute of Industrial Research, Pittsburgh, Pa. Because of a limited supply of these substances we were able to carry out only one experiment on each. We also tried ethyl apocupreine HCl (M.I. No. 15) but did not succeed in raising the plasma concentration above 4.12 mgm. per liter at which level no effect was observed.

† The quinine methochloride was provided by the Merck Co., the ethochloride by Parke Davis and Co.

RESULTS. The relative effectiveness of these compounds, taking as a basis for comparison the plasma concentration necessary to cause complete vagus block, is shown in table 1. These results are derived from experiments on forty dogs. Quinidine is the most effective of the four alkaloids, being about twice as potent as cinchonine and cinchonidine and about three times as potent as quinine in terms of effective plasma concentration. Of the three quaternary base derivatives quinine ethochloride is about twice as potent as quinine methochloride which has about the same effectiveness as quinine. The M.I. No. 102 compound, which has the p-hydroxyacetaminophenyl group and the chloride attached to the quinuclidine nitrogen, shows a potency close to that of quinine ethochloride. The effectiveness of the cupreine derivatives is lost in the hydroxy compound (M.I. No. 72). All of the active drugs caused some decrease in the vagus action at plasma concentrations considerably below those necessary to cause complete block.

In the above series the potency of the drug is defined in terms of effective plasma concentration rather than dosage. This seems a better basis of comparison since dosage is only one of the factors contributing to the maintenance of suitable concentrations at the site of action. The plasma concentration existing at any time represents a balance between those forces tending to increase it (in this case depending only on dosage) and those tending to reduce it such as binding or destruction by tissues, distribution through body fluids and renal excretion. When infusions are continued long enough to allow distribution through body fluids and establishment of a concentration plateau in the plasma, the rates of infusion are roughly equal to the rate of loss by metabolic destruction plus excretion at the given plasma level. In table 2 we have selected data

TABLE 2

Rates of infusion necessary to maintain plasma concentration plateaus at levels partially or completely blocking vagus inhibition of the heart. The effect on blood pressure is also indicated

DRUG	PLASMA CONC.	INFUSION		MEAN BLOOD PRESSURE	
		Rate	Duration	Control	Experimental
	mgm /liter	mgm /kgm /min	min		
Quinidine sulfate.	6.2	0.26	110	110	110
Cinchonine sulfate	12 3	0 88	60	115	65
Cinchonidine sulfate .	11.3	0 8	45	150	110
Quinine sulfate	23 6	0 77	210	110	95
Quinine methochloride	14 2	0.13	165	120	115
Quinine ethochloride	7 0	0 09	130	130	100
p-hydroxyacetaminophenyl-hydroxyethyl-apocupreinium HCl Chloride (M.I. No. 102)	13 3	0 068	100	120	100
n-butyl apocupreine 2 HCl (M.I. No. 76)	6.7	0.164	70	120	120
Ethyl dihydrocupreine (M.I. No. 0)	8.6	0.4	140	110	60

from experiments where such a concentration plateau had been established at plasma concentrations adequate to markedly decrease the effect of peripheral vagus stimulation on the heart. An examination of the rates of administration needed to maintain a given plasma level of the various drugs shows that there are marked differences in the rate of their removal from the blood. It is particularly noticeable that the quaternary derivatives are lost from the blood at a much slower rate than the alkaloids.

Much smaller total doses of quaternary salts than of the other compounds were required to achieve a given plasma level. This is probably due not only to their slower rate of loss but also to the fact that they do not readily enter tissue cells (unpublished observations) as do the alkaloids (9). The alkaloids have a large concentration reservoir in the tissues which tends to maintain the concentration in the plasma at effective levels for some time after the infusion has been stopped This difference in distribution probably accounts in large part for the fact that the blocking action of the alkaloids on the vagus persists

for a long time after the infusion has been stopped while the effect of the quaternary derivatives is readily reversible.

It is important to note that these animals continued to respire spontaneously, even, in some cases, with both vagi sectioned, at plasma concentrations of the drugs which blocked the vagus endings. This demonstrates that the vagus neuromuscular junctions are paralyzed at a plasma concentration below that at which skeletal neuromuscular junctions are blocked. A few experiments were carried out with quinine methochloride and quinine ethochloride to determine the plasma concentration necessary to cause general paralysis. This was found to be at least twice the concentration required to paralyze the vagus endings.

All of these drugs depressed the blood pressure when administered intravenously, (table 2) the degree of this depression being roughly proportional to the concentration of the infused solution. This effect of intravenous quinine is well known (5).

Although Gruber, et al. (6) have reported that barbiturates can cause a depression of the cardiac vagus nerves, there was little evidence of this effect in our experiments. In general the decerebrated dogs, (which were allowed an hour to recover from the ether anethesia before use) required about the same amount of the cinchona drugs as the dogs anesthetized with pentobarbital sodium.

DISCUSSION. There is evidence to indicate that the cinchona alkaloids exert a blocking action on all neuromuscular junctions. Oester and Maaske (12) made a detailed study of the effect of quinine on the skeletal neuromuscular junction of the dog including the antagonism of quinine to the effects of acetyl choline. They refer to the pertinent preceding literature. Ravin (13) has subsequently studied the action of quinine on the responses of skeletal muscles on cats. That the alkaloids may also depress the vasomotor activity of the sympathetic nerve endings is indicated by the work of Nelson who observed that quinine and quinidine given intravenously not only caused a drop in blood pressure, but antagonized the pressor effect of epinephrine. Nelson found, however, that some other motor effects of sympathetic stimulation or epinephrine injection were not antagonized by quinine (11). Chopra et al. (4) and Stake-Torsten (14) have also observed an antagonism of the cinchona alkaloids to the vasomotor effect of sympathetic nerve stimulation and epinephrine, while Stavraky (15) and Starr (16) noted a paralysis of parasympathetic endings and an antagonism to acetylcholine.

The curare-like paralytic action of the quaternary nitrogen derivatives of quinine on skeletal neuromuscular junctions is well known (3, 7, 10). It is apparent from our results that these agents also exert a paralytic effect on vagus neuromuscular junctions.

The cinchona alkaloids, with the exception of quinidine, exert their action on the vagus endings at concentrations which, if we can apply these results on the dog to man, are accompanied by toxic effects, possibly due in part to a loss of certain autonomic activities. It is interesting to note that the amount of quinidine usually given for auricular fibrillation might cause a plasma con-

centration adequate to block the vagus (8). As for the quaternary base derivatives tested, a dose adequate to produce general muscular relaxation would probably also paralyze the vagus.

That the lowered blood pressure is not responsible for the decreased effect of vagus stimulation is shown by the fact that vagus function often returned while the blood pressure was quite low. Furthermore, in some experiments the vagus was blocked without a marked lowering of the blood pressure. It might be expected that the paralysis of the vagus endings would cause an acceleration of the heart due to release from tonic vagus inhibition. However we found a decreased heart rate in most instances, probably due to a direct depression of the myocardial excitability (17). This slower heart rate contributed to the depressor effect on the blood pressure but the principal cause of the latter was probably a peripheral vasodilation (11), (5).

SUMMARY

1. The four principal cinchona alkaloids, three quaternary nitrogen derivatives, and three cupreine derivatives have been investigated with regard to the plasma concentration necessary to block the cardio-inhibitory action of maximal peripheral vagus stimulation in decerebrate and anesthetized dogs.

2. Of the cinchona alkaloids quinidine was effective at the lowest plasma concentration, causing a complete vagus block at concentrations averaging 7.4 mgm. of the sulfate per liter of plasma. Cinchonidine and cinchonine sulfate were effective at average concentrations of 15.5 and 13.3 mgm. per liter respectively, while quinine sulfate was least effective, requiring an average of 19.8 mgm. per liter. Quinine methochloride blocked the vagus at about the same concentration as quinine but quinine ethochloride was effective at about half this concentration. Three other derivatives of cupreine blocked the vagus in the same range of concentration but hydroxy ethylapocupreine showed no effect up to 14.9 mgm. per liter. All of the active drugs decreased the cardio-inhibitory effect of vagus stimulation at plasma concentrations considerably below those required to cause complete vagus block.

3. All of these agents caused a depression of blood pressure, the degree of this effect being closely related to the concentration of the infusion fluid. It is in part due to a slowing of the heart rate.

4. The possibility that these agents exert a blocking action on all peripheral neuromotor junctions, autonomic as well as skeletal, is considered.

5. Data on the rates of intravenous administration of these drugs necessary to achieve effective plasma concentrations are presented with a discussion of the factors involved.

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ISOPROPYL ALCOHOL

Acquired Tolerance in Dogs, Rate of Disappearance from the Blood Stream in Various Species, and Effects on Successive Generation of Rats[1]

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In previous communications the acute and chronic toxicity of isopropyl alcohol in experimental animals and the rate of metabolism in dogs were reported (1, 2). The present paper deals with observations on the effects of habitual consumption of isopropyl alcohol in dogs, the relationship between dosage and rate of fall of blood alcohol in various species, and the effects of continued drinking on successive generations of white rats.

I. Acquired tolerance to isopropyl alcohol. The occurrence of tolerance to isopropyl alcohol in animals has been reported (3). The statement has been made that rabbits do not become tolerant (4). A possible explanation was offered that the mechanisms involved are an accumulation of the alcohol due to slow detoxicating and production of a depressant metabolite such as acetone.

Because of the case with which isopropyl alcohol can now be obtained, some hazard from habitual consumption does exist and increased toxic manifestations as a result of regular or frequently repeated consumption could be a serious complication. No delayed toxic action was noted in rats which consumed the alcohol continuously for six months (1). However, under these circumstances detoxication may have kept pace with intake, a condition which might not obtain if inhibition were more intensive for short periods daily.

This phase of the problem had a two-fold purpose: to ascertain whether an acquired tolerance to isopropyl alcohol can develop in the dog, and whether chronic intoxication is distinct from acute poisoning.

Each of three adult dogs was given a test dose of isopropyl alcohol intravenously. The alcohol was diluted to a concentration of 20 per cent in normal saline solution (0.9 per cent sodium chloride) and given at the rate of 0.25 cc. per kilogram per minute by gravity into a leg vein. Dogs 1 and 2 received 3.84 cc. and dog 3 received 2.56 cc. of absolute alcohol per kilogram. These quantities represented $\frac{3}{4}$ and $\frac{1}{2}$ of the acutely fatal intravenous dose (1). Blood isopropyl alcohol determinations were made as previously described (2) on blood from a leg vein taken at appropriate intervals after completion of the injections. The degrees of drunkenness were recorded for each animal at the same time employing Newman's scale (5). After these control determinations were made the habituation period

was begun by placing the dogs on a fluid intake consisting solely of a solution of isopropyl alcohol and water, beginning with a 1 per cent concentration which was gradually increased to 4 per cent during the course of one month. Drinking was permitted only for one hour in the morning in order to insure daily high concentrations of blood alcohol. They were maintained on this regimen for 6 months. Under these conditions the animals became definitely inebriated once a day to about stage 4 of Newman's scale, that is, they were able to walk briskly, but were grossly ataxic. During the 6 months' period the dogs imbibed an average of 15.5 cc. of isopropyl alcohol daily, which represented 1.6 cc. per kilogram of body weight. Since isopropyl alcohol has been shown to possess about twice the depressant potency of ethyl alcohol, the animals drank an amount of the alcohol which was the equivalent of a pint of 90 proof whiskey daily for a 70 kilogram man.

During the period of habituation, and at intervals of approximately 2 weeks, blood isopropyl alcohol determinations were made 1 hour after the daily imbibition. These values, varying from 64 mgm. per cent or 114 mgm. per cent, correlated well with the concentrations found in dogs which received $\frac{1}{4}$ of the acutely fatal gastric dose, or 1.87 cc. per kilogram. The only physical deterioration which was observed occurred in dog 1 in which a weight loss of 1.5 kgm. from the initial weight of 10 2 kgm. was noted.

At the end of the habituation period the test dose was repeated and the animals subjected to the same procedure as before. When these observations were completed the dogs were sacrificed and submitted for pathologic study with particular attention to the gastrointestinal tract and the central nervous system.

Prolonged imbibition and tolerance. Figure 1 presents the blood isopropyl alcohol curves and degrees of intoxication for the individual dogs before and after habituation. It may be seen that the concentration of the alcohol in the blood at the end of the first hour in the abstinent animals following the test dose is almost coincidental with that found after habituation. Significant increase in the rate of fall of blood alcohol appeared in the second hour and although there was some variation among the individual dogs the slopes of the curves throughout the period of observation were more acute for the animal after habituation than before. The application of Widmark's formula (7) to the metabolic rates also emphasizes this fact In dog 1 the alcohol was removed from the blood stream 110 per cent faster than before habituation. Dog 2 showed an increase of 4 per cent and dog 3 an acceleration of 44 per cent over the rate established in the abstinent state.

The degree of drunkenness after habituation showed a consistent decrease in all dogs especially after the third hour. Dog 3 demonstrated this to a greater extent than the other animals. There is probably some correlation between the extent of neuromuscular coordination and increased rate of fall of blood isopropyl alcohol after habituation, but when the blood alcohol concentrations and degrees of drunkenness are compared in dog 2 the correlation is not so marked. The development of an acquired tolerance offers the only reasonable explanation of the changes seen.

Pathologic changes in viscera and brain. Dog 1 was found dead 36 hours after completion of the second test dose procedure. Dogs 2 and 3 were sacrificed at this time and Dr. M. E. Maun, of the Department of Pathology of Wayne University, examined sections of the heart, blood vessels, liver, kidneys, spleen, stomach, duodenum and ileum. The gastrointestinal tract of all animals

showed no evidence of pathologic changes. The significant changes in the other organs were limited to the kidneys of dog 1. These tissues revealed a
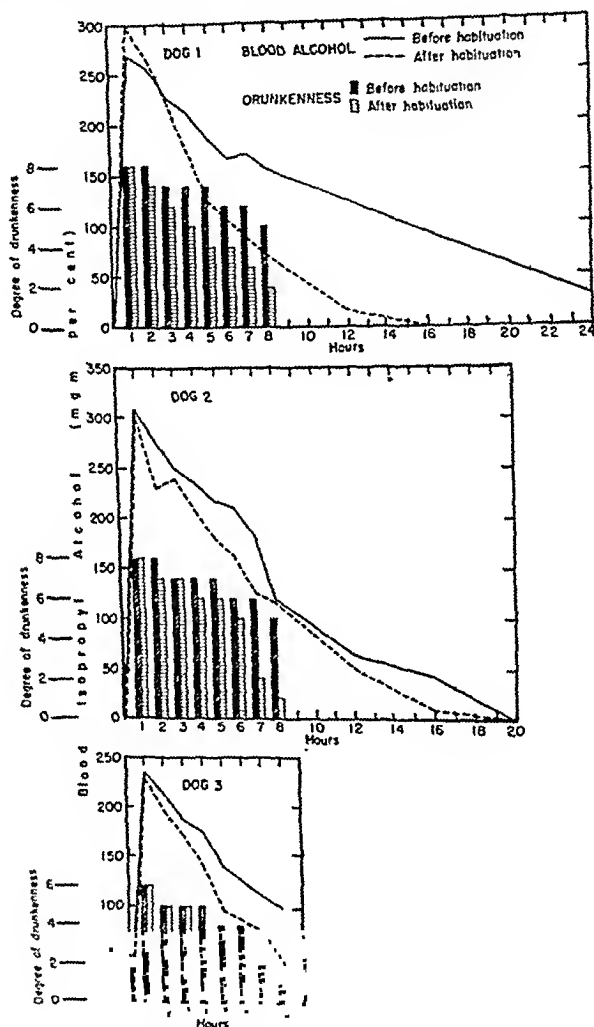

FIG. 1 RELATIONSHIP BETWEEN BLOOD ISOPROPYL ALCOHOL AND DEGREES OF DRUNKEN-NESS IN ABSTINENT AND HABITUATED DOGS

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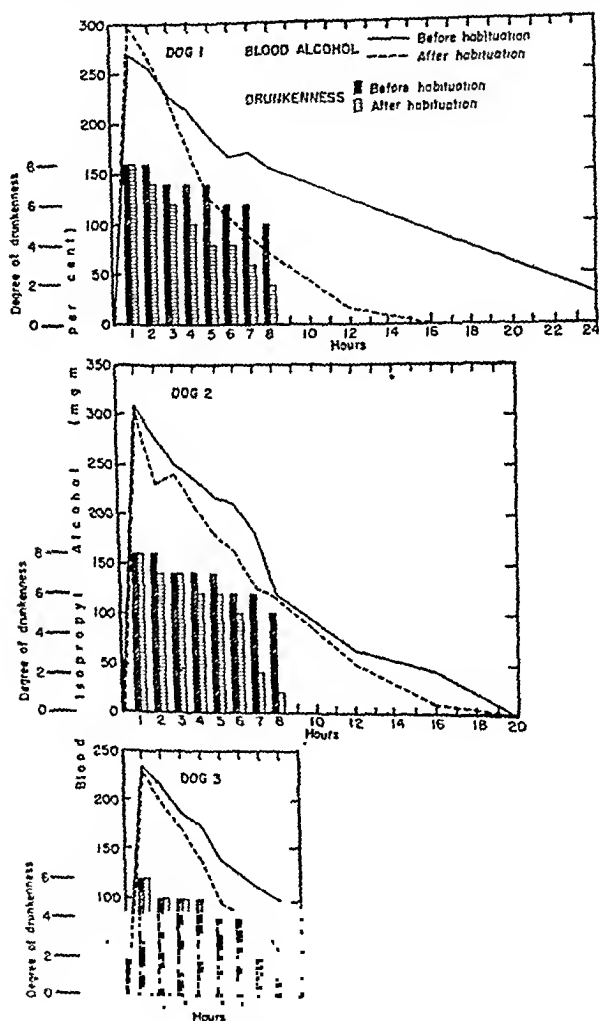

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decrease in the number of nephrons with hydropic changes and necrosis of some of the tubular epithelium.

The brains were examined by Dr. Gabriel Steiner, of the Brain Disease Registry of Wayne University. The brain of dog 1 showed a subcortical pale spot

with a small central hemorrhage, leucodiapedesis and microglial reaction. In dog 2 a few small capillary hemorrhages in the walls of the third ventricle were seen. No abnormal changes were found in the brain of dog 3.

The structural defects which may have caused the death of dog 1 could not be ascertained. It would appear that nervous tissue is more sensitive to the actions of isopropyl alcohol than are the kidneys.

II. RATE OF DISAPPEARANCE OF ISOPROPYL ALCOHOL FROM THE BLOOD STREAM IN VARIOUS SPECIES. The rate of decline of isopropyl alcohol in the blood stream in dogs was shown to be dependent upon alcoholic concentration (2). A low dose invariably resulted in a slower decline than did a high dose. Similar studies with ethyl alcohol in several species showed that the alcohol metabolism proceeded at a constant rate regardless of the dosage (6). It was of interest, therefore, to determine whether or not the variable decrease in alcoholemia found in the studies of isopropyl alcohol in dogs was peculiar to that species or a common phenomenon among vertebrates.

The observations were extended to include the following animals: cat, rabbit, rat and pigeon. For the purpose of comparison under similar conditions a series of observations was repeated on dogs. Each species was divided into two groups of three animals each, one group receiving a low dose, or 0 987 grams (1.25 cc.) per kgm., the other receiving a high dose, or 1.974 grams (2 5 cc.) per kgm. The alcohol was given intravenously and one hour was allowed after injection for equilibration between blood and tissues before blood samples were taken for analysis. Blood alcohol concentrations were followed at hourly intervals for six hours, with blood taken from the heart in rabbits and pigeons and from peripheral veins in dogs and cats Because of their small size, a group of 18 rats each was used for the low and high dose schedules. One-third of each group was sacrificed at hourly intervals one hour after injection and a sample of blood taken from the heart. The factor r which represents the mean alcohol concentration of the whole body expressed as a fraction of the concentration in the blood, Beta, or rate of decline of blood alcohol concentration in mgm. per gram of blood per minute, and c_0, or the theoretical concentration of isopropyl alcohol in the blood immediately after injection, were determined by the method devised by Widmark (7) for ethyl alcohol. The data for each species are presented in table 1, with a summary of averages in table 2.

A significant feature of these tables is that the factor r for rabbits, rats and pigeons exceeds unity. This indicates that either there is initially a very rapid metabolism of the alcohol, or that there is a storage in the tissues in a concentration greater than appears in the blood stream. The first of these concepts is supported by both the higher r values for the low dose as compared with the high dose, and Beta which is larger for the high dose than for the low dose. Further confirmatory evidence for the acceleration hypothesis is found in a study of distribution of isopropyl alcohol in the body tissues of dogs (8). The high concentrations of the alcohol which were found in blood and heart muscle as compared with the other tissues examined 4 hours after the animals had received approximately 4 cc. per kilogram by stomach is not in accord with the

TABLE 1

The values for c_0, r and Beta in various species after intravenous injection of isopropyl alcohol

SPECIES	DOSE	c_0	r	BETA
Dog	gm./kg.			
	0.987	1.06	0.93	0.0022
	0.987	0.99	1.00	0.0020
	0.987	1.00	0.98	0.0020
	1.974	2.88	0.69	0.0043
	1.974	2.10	0.94	0.0033
	1.974	2.22	0.89	0.0045
Cat	0.987	1.23	0.80	0.0019
	0.987	1.02	0.93	0.0024
	0.987	1.25	0.79	0.0018
	1.974	2.25	0.88	0.0019
	1.974	2.17	0.91	0.0022
	1.974	2.30	0.85	0.0029
Rabbit	0.987	0.66	1.49	0.0017
	0.987	0.76	1.30	0.0019
	0.987	0.92	1.07	0.0019
	1.974	1.89	1.04	0.0030
	1.974	1.55	1.27	0.0025
	1.974	1.80·	1.09	0.0034
Rat	0.987	0.66	1.49	0.0016
	0.987	0.88	1.12	0.0023
	0.987	0.73	1.35	0.0020
	1.974	1.68	1.17	0.0036
	1.974	1.53	1.29	0.0027
	1.974	1.45	1.36	0.0032
Pigeon	0.987	0.94	1.05	0.0039
	0.987	0.63	1.57	0.0026
	0.987	0.83	1.19	0.0034
	1.974	2.03	0.97	0.0054
	1.974	1.75	1.13	0.0047
	1.974	1.45	1.36	0.0039

TABLE 2

Summary of average values for r and Beta

SPECIES	r		BETA	
	Low dose	High dose	Low dose	High dose
Dog	0.97	0.84	0.0021	0.0041
Cat	0.84	0.88	0.0020	0.0023
Rabbit	1.29	1.13	0.0018	0.0026
Rat	1.32	1.27	0.0020	0.0032
Pigeon	1.27	1.15	0.0033	0.0047

thesis of tissue storage.   The values for r and Beta for the dog show tendencies similar to those observed in rodents and fowls.   Beta in this instance was found to be almost twice as great for the high as for the low dose.   There is apparently a difference between the cat and the other species as regards the fate of the injected alcohol.   Beta is about equal for both dosage schedules which would indicate a constant rate of metabolism regardless of the dose used.   As might be expected, Beta is significantly greater in pigeons than in mammals.

III. EFFECTS OF CONTINUED VOLUNTARY DRINKING OF ISOPROPYL ALCOHOL ON SUCCESSIVE GENERATIONS OF WHITE RATS.   It has been demonstrated that ethyl alcohol can have detrimental effects on the reproductive functions and embryonic development in guinea pigs (9).   This was especially pronounced when alcoholic individuals were mated.   To ascertain the influence of isopropyl alcohol on reproduction and growth in mammals the procedure of voluntary drinking was adopted and studied in white rats.   Offspring from the control animals employed in the original chronic toxicity experiments (1) were used.   Six females and 3 males were selected and when 38 to 40 days old the segregated sexes were placed on the standard diet and 2 5 per cent isopropyl alcohol in the drinking water.   When the rats were 120 days old they were mated according to the Wistar method (10).   This was repeated through 2 generations.   Selection of the 2.5 per cent concentration of the alcohol was based on preliminary experiments as being the most satisfactory for the purpose.   When nursing young reached the age when additional fluid intake became necessary the animals refused to drink concentrations higher than 2 5 per cent and invariably died of dehydration.

RESULTS.   The six matings produced 4 litters totaling 44 young.   The mating of 13 females in the first generation produced 11 litters of from 4 to 12 rats each, or a total of 66 second generation offspring.   Two groups of 10 males and females each of approximately equal weight and age were selected from the latter.   Five animals from each group were placed on water and their growth rate compared with the alcoholized rats.   Growth curves of the several series of rats were charted, using the log of the weight and the reciprocal of time as coordinates (11).   Figure 2 presents a summary of the data.

It is seen that there is no significant difference in the rate of growth of second generation alcoholized rats as compared with a similar group placed on water and control non-alcoholized animals.   There was some retardation of growth in the first generation offspring of alcohol-fed rats during the early weeks of life but they were rapidly approaching the controls by the 13th week.

The daily isopropyl alcohol intake per kilogram of the original mothers and fathers was 1 87 cc , that of the first generation, 1.76 cc , and of the second generation, 1.64 cc.   The nursing young were also imbibing the alcohol through the mother's milk as demonstrated by analysis of various organs from 5 first generation animals which were sacrificed when 20 days old   The combined livers yielded 52.5 mgm. per cent; the stomach, 78 0 mgm. per cent; and the brains, 4.7 mgm. per cent.   It seems reasonable to assume therefore that, with the exception of some retardation of growth early in the life of first generation

rats, isopropyl alcohol comprising 2.5 per cent of the fluid intake does not produce any deleterious effects on the reproductive functions and embryonic development in white rats.
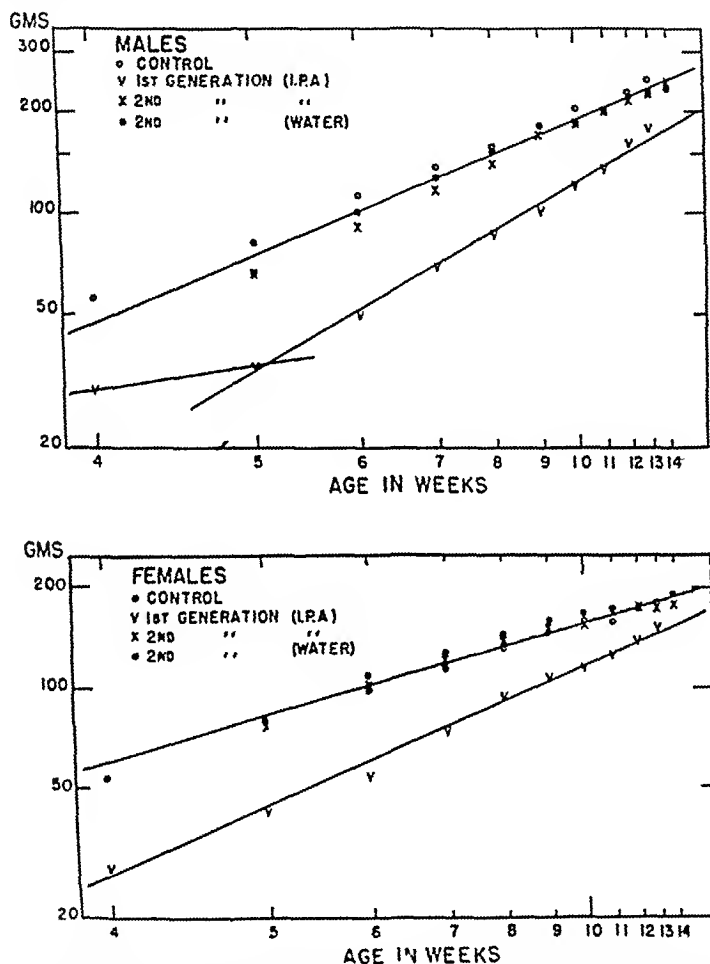
FIG. 2. EFFECT OF CONTINUED VOLUNTARY DRINKING OF 2.5 PER CENT ISOPROPYL ALCOHOL (I.P.A.) IN WATER ON BODY WEIGHT OF SUCCESSIVE GENERATIONS OF WHITE RATS

DISCUSSION. The toxicity of isopropyl alcohol has been likened to that of methyl alcohol in which the protracted and cumulative action is ascribed to slow metabolism and excretion. It is evident from the data on the rate of metabolism in the various species that isopropyl alcohol is disposed of rapidly and does not

remain in the blood stream much beyond 24 hours even with quantities as large as ¾ of the acutely fatal intravenous dose administered to dogs.   Likewise the concept of cumulative tendencies and production of harmful intermediate metabolites is not supported by the facts herein reported.   The habituation studies in dogs demonstrated that large quantities of isopropyl alcohol can be imbibed over short periods daily without evoking cumulative actions.   These animals which were definitely inebriated 3 to 5 hours daily for one-half year fared well on a standard dry diet and 4 per cent alcohol.   At the beginning of each drinking bout the behavior of the dogs was that of any normal healthy animal.   Furthermore, the studies of alcoholism in mammals have shown that although ethyl alcohol administered daily to guinea pigs does not greatly disturb the physiologic processes, a marked influence is exerted on the germ cells and developing embryos.   The absence of any effect of isopropyl alcohol on the reproductive functions on successive generations of white rats voluntarily drinking concentrations up to the limit of tolerance for very young animals would argue against a delayed toxicity or the production of harmful intermediate metabolites.

Further consideration of the problem of isopropyl alcohol metabolism reveals that there is a relationship between dosage and rate of oxidation.   Taking the average values for Beta for dogs, rabbits, rats and pigeons it is seen that doubling the dose increased the rate of fall of blood isopropyl alcohol by 95 per cent, 44 per cent, 60 per cent, and 42 per cent, respectively.   Cats do not show this response to the same degree as do the other species.   Actually, a 15 per cent increase was noted.   It is equally evident that no direct proportionality between the blood alcohol concentration and rate of disappearance exists, since the average blood alcohol concentration for the high dose in each species is approximately twice that for the lower dose, while the higher rates of metabolism vary from 15 per cent in cats to 95 per cent in dogs.

A comparison of the metabolic rates of ethyl and isopropyl alcohol also shows that these two substances must be detoxified by different mechanisms.   The curve of blood ethyl alcohol concentration plotted against time is linear in form, although the rate is dependent to some extent on the dosage.   Each doubling of the dose increases rate of fall by approximately 17 per cent (12).   In contrast to this the blood isopropyl alcohol curves show considerable deformations and are exponential in character.   Calculations of actual differences of the two alcohols in the several species for which data are available show that ethyl alcohol is more rapidly oxidized than is isopropyl alcohol.   The value for Beta in dogs on the high isopropyl alcohol dose is the only exception.   Taking the highest average values for Beta for the various species in the isopropyl series and calculating the percentage difference for the two alcohols, it is found that dogs metabolize isopropyl alcohol approximately 57 per cent faster than ethyl alcohol. On a similar basis, cats burn ethyl alcohol 39 per cent faster; rabbits 46 per cent faster; and pigeons 8 per cent faster.   In general this supports the contention of other investigators that the destruction of isopropyl alcohol in the body is slow.

CONCLUSIONS

1. Prolonged imbibition of large amounts of isopropyl alcohol daily for a period of 7 months renders dogs tolerant to the alcohol. This tolerance was manifested by a greater degree of neuromuscular coordination at similar blood alcohol concentrations after habituation than before, and by an increased elimination of the alcohol. A careful study of the structural defects of important viscera from the animals sacrificed after the period of habituation indicated that the central nervous system may be more sensitive to the action of isopropyl alcohol than other tissues, but kidneys may be damaged.

2. The rate of isopropyl alcohol metabolism after intravenous administration in dogs, cats, rabbits, rats, and pigeons as determined from post-absorptive blood levels is dependent on the dosage. The elimination of the alcohol on a low dose is most rapid in the pigeon, somewhat slower in the other 4 species. On a high dose elimination is most rapid in the pigeon, somewhat less rapid in the dog and rat, and least rapid in the rabbit and cat. It is suggested that the rate of disappearance may be dependent upon physiologic mechanisms peculiar to each species rather than upon metabolic rate and ratio of surface area to weight.

3. Continued voluntary drinking of 2.5 per cent isopropyl alcohol through 2 successive generations of white rats produced no effect on reproductive functions and embryonic development. The progeny derived from the treated animals showed no deviations from the normal. The growth of the first generation offspring was retarded during the early weeks of life but essentially normal growth was established by the 13th week.

4. The ingestion of large amounts of isopropyl alcohol in dogs at regular daily intervals failed to evoke any evidence of cumulative action. No secondary toxic manifestations as a result of harmful intermediate metabolites were observed, and it would appear that chronic toxicity differs in no way from acute poisoning.

5. Isopropyl alcohol is metabolized at a slower rate than is ethyl alcohol. Dogs on a high dose offer the only exception. The curves of post-absorptive blood isopropyl alcohol concentrations are exponential in character as contrasted with those of ethyl alcohol which are linear.

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FURTHER OBSERVATIONS ON THE PRESSOR ACTION OF OPTICAL ISOMERS OF SYMPATHOMIMETIC AMINES

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In a previous communication (1), it was reported that the *l*-isomers of desoxy-ephedrine, benzedrine, *iso*benzedrine, propadrine, and pseudo-propadrine are more potent in raising blood pressure than the *d*-isomers. It was also shown that the *l*-mandelates of *l*-benzedrine and *d*-*iso*benzedrine are more powerful than the *d*-mandelates of *l*-benzedrine and *d*-*iso*benzedrine, respectively, indicating the favorable influence of *l*-mandelic acid on blood pressure. The latter is particularly interesting and merits further investigation. In order to extend the study, additional sympathomimetic amines were employed and the mandelates prepared. The compounds are listed in table 1.

TABLE 1
Comparison of pressor action in pithed dogs

COMPOUND	M P.	SPECIFIC ROTATION		DOSE RANGE TESTED	NUMBER OF DOGS USED	GEOMETRICAL MEAN OF EPINEPHRINE EQUIVALENT OF 1 MG. ± STANDARD ERROR
	°C			*mg.*		*μg.*
l-Ephedrine *l*-mandelate.. . .	169–170	$[\alpha]_D^{25}$	−72.6°	5–15	8	2.36 ± 0.26
l-Ephedrine *d*-mandelate . . .	100–106	$[\alpha]_D^{25}$	+23.6°	6–15	8	1.99 ± 0.23
β-Phenylethylamine *l*-mandelate.	127–128	$[\alpha]_D^{25}$	−66.5°	3–10	9	3.49 ± 0.32
β-Phenylethylamine *d*-mandelate	126–127	$[\alpha]_D^{25}$	+66.0°	5–15	8	3.67 ± 0.38
Tyramine *l*-mandelate	159–160	$[\alpha]_D^{27}$	−40.0°	4–20	6	4.32 ± 0.52
Tyramine *d*-mandelate .	166–167	$[\alpha]_D^{27}$	+65.0°	5–14	5	4.32 ± 0.55
l-Mandelic acid..	133–134	$[\alpha]_D^{27}$	−150.0°	20–60	5	0
d-Mandelic acid...	133–134	$[\alpha]_D^{27}$	+150.0°	20–60	5	0
l-2-Aminoheptane sulfate .	245–247	$[\alpha]_D^{25}$	+2.7°	5–14	7	2.19 ± 0.20
dl-2-Aminoheptane sulfate.. .	250*			5–12	5	3.06 ± 0.14
d-2-Aminoheptane sulfate .	244–247	$[\alpha]_D^{20}$	−2.2°	5–10	7	4.21 ± 0.23
d-2-Aminoheptane *d*-mandelate	137–139	$[\alpha]_D^{22}$	+62.0°	6–12	5	3.15 ± 0.24
d-2-Aminoheptane *l*-mandelate ..	121–122	$[\alpha]_D^{27}$	−66.0°	6–15	5	1.76 ± 0.20
l-2-Aminoheptane *l*-mandelate..	137–139	$[\alpha]_D^{25}$	−62.5°	10–20	5	1.28 ± 0.25
l-2-Aminoheptane *d*-mandelate .	116–117	$[\alpha]_D^{25}$	+66.0°	14–20	5	0.95 ± 0.17

* Beginning to decompose at this temperature.

The first 8 compounds were made according to the method of Jarowski and Hartung (2) by one of us (F. A. S.). The remaining 9 compounds were kindly supplied by Drs. Ewald Rohrmann and Ming-Chien Chiang. The melting points and specific rotations have also been recorded, as shown in the table. The synthesis of aminoalkanes, including 2-aminoheptane, has been published by Rohrmann and Shonle (3). The method of estimating the pressor action of

FIG. 1. COMPARISON OF PRESSOR ACTION IN PITHED DOGS

A, B, C, and D designate results from 4 different dogs. All the numbers on the left-hand side of each tracing indicate the doses of epinephrine in μg., injected intravenously.

each substance in pithed dogs was the same as previously described (1). A 2% solution was used in each case. Doses varied according to the activity of the compound and the sensitivity of the dog. Injections were all made intravenously.

The plan of study was to ascertain whether or not (a) *l*-mandelates of other amines were always more potent than the *d*-mandelates of the same isomers; (b) *l*- or *d*-mandelic acid had any pressor action by itself; and (c) optical isomers of aliphatic amines showed the same pattern as the aromatic amines.

The results of the pressor action of the present series are shown in table 1. Since all compounds exhibit, to a more or less degree, tachyphylaxis, groups of 5 to 9 dogs were individually tested for each isomer against epinephrine. It should be observed that *l*-ephedrine *l*-mandelate has a predominant tendency of being stronger than *l*-ephedrine *d*-mandelate, confirming our previous data (1). However, the *l*-mandelates of optically inactive bases, such as, β-phenylethylamine and tyramine, showed no superiority to the *d*-mandelates. The difference between β-phenylethylamine *l*-mandelate and β-phenylethylamine *d*-mandelate was less than 1 standard error, while the values for tyramine *l*-mandelate and tyramine *d*-mandelate were identical. Both isomers of mandelic acid are inactive. It appears that among the aromatic amines it requires optical activity of the amine in order to elicit the increase of action by *l*-mandelic acid on the blood pressure.

With 2-aminoheptane sulfate, an interesting feature appeared; namely, the *l*-isomer has a dextrorotation in aqueous solution; and the *d*-isomer, a levorotation. This has been known as the "acid-effect" (4), although the explanation of the reversal of optical rotation is not as yet available. Equally interesting is the fact that *d*-2-aminoheptane sulfate is almost twice as active as its *l*-enantiomorph. An example of comparison is given in figure 1, A and B. The racemic mixture, that is, *dl*-2-aminoheptane sulfate, is intermediate in its pressor action, as shown in table 1. In contrast to the sulfates, the *d*-mandelate of *d*-2-aminoheptane, dextrorotatory in water, is decidedly more powerful than the *l*-mandelate of *d*-2-aminoheptane (see figure 1, C and D). Also, the *l*-mandelate of *l*-2-aminoheptane, levorotatory in water, surpasses the activity of the *d*-mandelate of *l*-2-aminoheptane, the difference being greater than 1 standard error. These data further demonstrate that the optically active mandelic acids when neutralized with optically active sympathomimetic amines to form salts are capable of enhancing the pressor activity of the latter. The explanation for such modification of action must be left for the future.

<div align="center">SUMMARY</div>

1. Of the sympathomimetic drugs, the *l*-mandelate of optically active aromatic amines appears to be more active in raising the blood pressure of pithed dogs than the *d*-mandelate of the same isomer, as exemplified by *l*-ephedrine and other previously reported compounds.

2. No difference in pressor action can be detected between the enantiomorphous mandelates of optically inactive amines, such as β-phenylethylamine and tyramine.

3. Neither *l*- nor *d*-mandelic acid raises blood pressure.

4. Of the 2-aminoheptane sulfates, the *d*-isomer having levorotation in water is almost twice as active as the *l*-isomer, which has a dextrorotation, the *dl*-isomer being intermediate between the two.

5. Differences in pressor action can be also detected between the enantiomorphous mandelates of *d*-2-aminoheptane and those of *l*-2-aminoheptane.

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THE HYDROLYSIS OF DEMEROL BY LIVER IN VITRO

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Demerol is the ethyl ester of 1-methyl-4-phenyl isonipecotic acid. It is probable that hydrolysis of the ester occurs in the body and that this is part of the mechanism by which the drug is inactivated. It was therefore of interest to study the hydrolysis *in vitro* in order to determine the distribution, properties, and specificity of the enzyme involved.
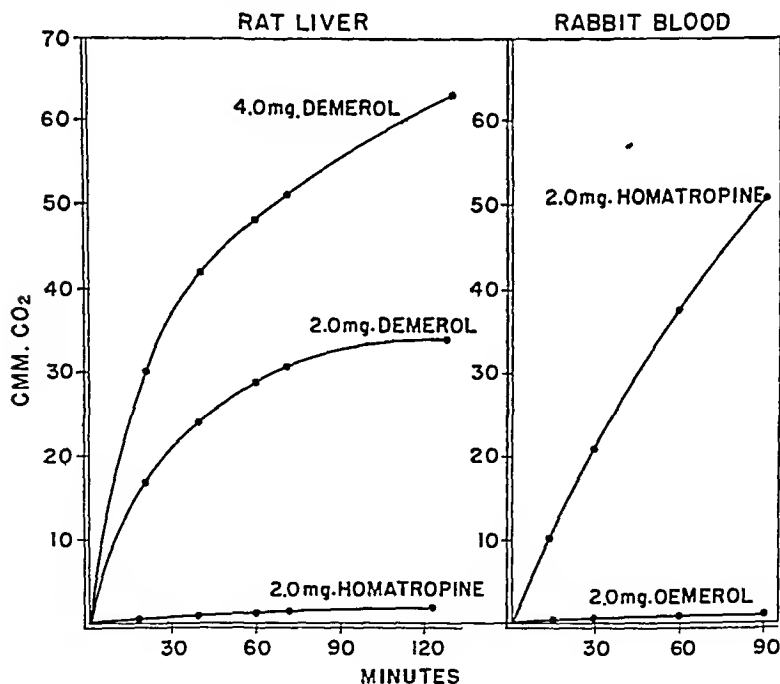
Fig. 1

EXPERIMENTAL. The demerol was supplied by the Winthrop Chemical Company. It is stable and shows no appreciable hydrolysis at pH 8.1 but begins to hydrolyze spontaneously at approximately pH 9.0. The isonipecotic acid formed is apparently a very weak acid with a pH near that of carbonic acid. Consequently it will not completely displace the theoretical amount of

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carbon dioxide from a solution of sodium bicarbonate in equilibrium with 5% carbon dioxide in the atmosphere. Despite this, the manometric method was used for these experiments, for it is more convenient and accurate than titri-

DOG LIVER
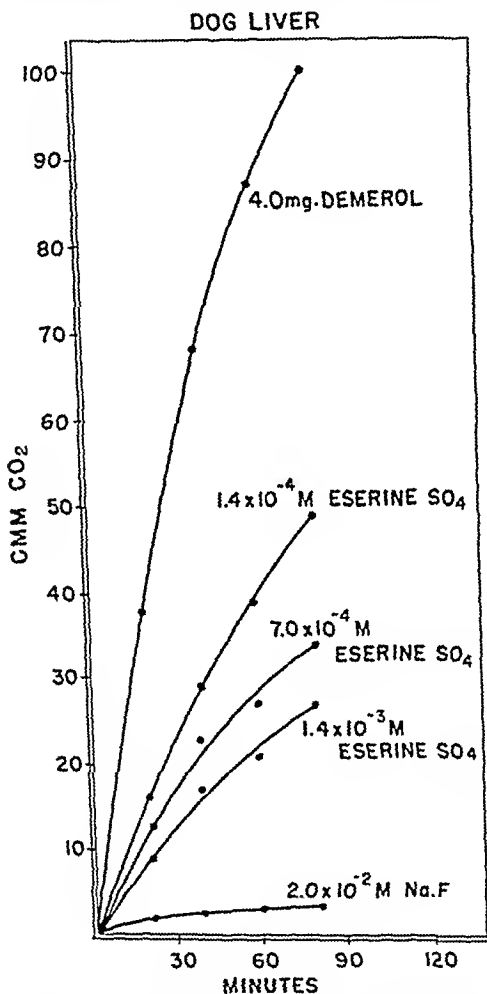
FIG. 2. THE EFFECT OF VARIOUS CONCENTRATIONS OF ESERINE AND OF SODIUM FLUORIDE ON THE HYDROLYSIS OF 4.0 MG. DEMEROL

metric methods. The ground tissue was suspended in M/20 phosphate buffer and M/20 NaHCO₃ and the vessels were filled with 95% nitrogen and 5% CO₂ and equilibrated before adding the drug. The final pH was 8.1. The increase in pressure due to the displacement of the carbon dioxide by the acid formed

was measured in the usual way in the Warburg manometers. Ethyl alcohol is the other product of hydrolysis. If small amounts of oxygen remained in the vessels it was possible that the alcohol would be oxidized by the alcohol oxidase present in certain tissues. In order to avoid this source of error cyanide was added. This completely inhibits the alcohol oxidase and has no effect on the course of the hydrolysis of demerol.

The enzyme is present only in the liver. No hydrolysis occurred when brain, blood, kidney, spleen, or heart tissue was used. It is present in the livers of puppies, rabbits, rats, guinea pigs, cats, turtles and frogs. This distribution differentiates the enzyme from those which hydrolyze atropine and homatropine for the latter are found only in guinea pig liver and rabbit liver and blood (1, 2). Figure 1 illustrates this point. Rat liver hydrolyzes demerol but not homatropine whereas rabbit blood hydrolyzes homatropine but not demerol. Figure 1 also shows the amount of carbon dioxide evolved is proportional to the amount

TABLE 1

The hydrolysis of 4.0 mg. demerol by different amounts of dog liver suspension at pH 8.1 and 37°

The figures are c.mm. carbon dioxide evolved. The control values have been subtracted

TIME	0 3 cc.	0 6 cc.	0 9 cc.
min.	*c m m*	*c m m.*	*c m m.*
20	30	50	57
40	42	60	69
60	47	72	81
80	56	85	96
105	68	92	102
165	86	100	115

of demerol present but the evolution always stops before the theoretical amount is attained. The rate of hydrolysis but not the end point is affected by the amount of tissue present. This is shown in table 1.

The activity of the enzyme is completely inhibited by $M/50$ sodium fluoride. Eserine in comparatively low concentrations also inhibits it. Figure 2 illustrates these facts. The sensitivity of the enzyme to eserine differentiates it from the simple esterases and from the mandelic acid esterases which are not inhibited by the concentrations of the drug that affect the demerol esterase. Stable preparations of the latter can be made if the tissue suspension is treated twice with 4 volumes of acetone and then dried. The enzyme is present in liver that has been perfused free of blood and if tissue suspensions are diluted with water and centrifuged, the enzyme is present in the supernatant fluid and is therefore associated with the more soluble proteins.

DISCUSSION. It is not possible at present to state what normal function the enzyme that hydrolyzes demerol performs. Its presence in the livers of all the animals tested indicates that it may be hydrolyzing esters with configurations similar to that of demerol. Its distribution shows that it is distinct from

the tropine esterases and the cholinesterases. The latter are inhibited by demerol (3). The enzyme's sensitivity to escrine distinguishes it from the simple esterases. It is becoming ever more apparent that there are specific enzymes present in tissue for every drug inactivated in the body.

SUMMARY

1. Demerol is hydrolyzed by the livers of various animals but not by any of the other tissues tested.

2. The hydrolysis is inhibited by eserine and by fluoride.

3. The enzyme responsible for the hydrolysis is distinct from the tropine esterases, cholinesterases, and the esterases which hydrolyze aliphatic esters.

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BIOLOGICAL COMPARISON OF LOCAL ANAESTHETICS

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There is at present no wholly satisfactory method for estimating the potency of local anaesthetics. It is generally realized that the method employed must vary according to the way in which the local anaesthetic will be applied and that the results obtained by different modes of application differ widely.

Sollmann's (1) method using plexus anaesthesia in frogs, gives an indication not only of the effective concentration but also of the power of penetrating nerve sheaths. It has been customary to determine the minimum effective concentration and the time taken for the onset of anaesthesia. Sinha (2) based his method on the duration of anaesthesia. He used the intracutaneous human wheal and found that there was an approximate linear relation between the logarithm of the concentration of the local anaesthetic used and its duration of action. McIntyre and Sievers (3, 4) used guinea-pigs for the intracutaneous wheal. The endpoint of local anesthesia is, however, hard to determine accurately. MacIntosh and Work (5) argued that the measurement of the intensity of anesthesia was more important than that of the duration, as this can be modified by adding adrenaline. They compromised by using an index based on both duration and intensity, trying to find the concentration necessary to produce full anaesthesia for a standard time of five minutes. Recently Chance and Lobstein (6) introduced a new method of using the guinea-pig's cornea. The corneal reflex was tested 6 times in succession at regular intervals and thus a certain number of responses out of six possible responses was obtained. This method provided a quantitative expression for a degree of anaesthesia less than complete anaesthesia.

In contrast to Chance and Lobstein we found the guinea-pig's intracutaneous wheal a better test than the corneal reflex. The guinea-pigs frequently fail to blink even if the cornea is not anaesthetised, but they give a prompt and reliable reaction to a light pin-prick of the skin. This is in agreement with the experience of MacIntosh and Work. We therefore applied the method of Chance and Lobstein to the intracutaneous wheal method in guinea-pigs, using several concentrations of the local anesthetic and thereby obtaining various degrees of anaesthesia instead of producing the maximum effect. Similarly we have developed a method based on plexus anaesthesia in frogs, making no attempt to estimate the minimum anaesthetic dose, but establishing a relation between different concentrations and their effects.

METHOD. 1. *Intracutaneous wheal in guinea pigs* Fully grown guinea-pigs are chosen. On the day preceding the experiment the hair on the back is clipped and two areas of 4–5 cm. diameter are shaved. This produces a certain amount of irritation which disappears overnight. The sensitiveness of the skin is greatest in the midline and

in the front area than in the back area. For this reason each concentration of a local anaesthetic must be tested in both areas. Six tests using three guinea-pigs can be performed simultaneously. The dose is always injected intracutaneously in 0.25 cc. saline. Three guinea-pigs receive one dose in the front area and another dose in the back area; the size of the wheal is marked with ink. The reaction to pin prick is tested five minutes after the injection in the following way. After observing the animal's normal reaction to a prick applied outside the wheal, six pricks are applied inside the wheal and the number of pricks is counted to which the guinea-pig fails to react. The pricks are applied at intervals of about 3–5 sec. The test of six pricks is applied every 5 minutes for 30 minutes.

TABLE 1

Number of pricks (out of six) failing to elicit a response after intracutaneous injection of nupercaine in guinea-pigs

TIME	0.0125%						0.025%					
	Back			Front			Front			Back		
	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Pig 6
min.												
5	5	6	6	5	3	4	6	6	6	6	6	6
10	3	4	2	2	3	4	3	6	5	3	5	6
15	3	2	2	0	0	3	3	2	5	2	3	4
20	1	0	1			0	3	1	4	0	3	4
25	0		0				1	0	0		3	3
30							0				0	0

Mean sum for 30 minutes 9.8 ±1.08   Mean sum for 30 minutes 17.5 ±1.76

TIME	0.05%						0.1%					
	Front			Back			Back			Front		
	Pig 7	Pig 8	Pig 9	Pig 10	Pig 11	Pig 12	Pig 7	Pig 8	Pig 9	Pig 10	Pig 11	Pig 12
min.												
5	6	6	6	6	6	5	6	6	6	6	6	6
10	4	6	6	6	5	4	6	6	6	6	6	6
15	4	5	5	6	5	4	6	6	6	6	6	6
20	4	4	3	6	5	4	6	6	6	6	6	6
25	1	4	2	6	4	3	6	6	6	6	5	6
30	1	1	1	2	3	3	5	6	6	6	3	6

Mean sum for 30 minutes 25.3 ±1.86   Mean sum for 30 minutes 35.2 ±0.66

The number of times the prick fails to elicit a response during the 30 min. period is added up and the sum, out of a possible 36, gives an indication of the degree of anaesthesia. Having completed the test on three guinea-pigs, the same solutions are injected into three other guinea-pigs, but the solution which was used for the front is now used for the back area and vice versa. An experiment shown in table 1 determining the relation between different concentrations of nupercaine and the degree of local anaesthesia may serve as an example. Table 1 shows that an average response from 6 tests was obtained for each concentration. After injecting 0.0125% nupercaine the guinea-pigs failed to respond to pin-prick only 10 times out of 36, whereas 0.025% produced 17 failures, 0.05% produced 25 failures and 0.1% produced almost full anaesthesia for 30 minutes.

2. *Plexus anaesthesia in frogs.* Rana temporaria are used which are kept in store at 4°C. No attention is paid to weight or sex. The frog is decapitated and the upper part of the spinal cord is destroyed down to the level of the third vertebra. A transverse incision is made in the abdominal wall just below the sternum. The viscera are removed through this opening carefully exposing the lumbar plexus without damaging it. The frog is pinned to a vertical board. The solution of the local anaesthetic dissolved in 0.7% saline is put into the pocket formed by the lower abdomen. The amount of solution used is irrelevant as long as the plexus is submerged. A record is made of the time taken to abolish the reflex contraction to a sensory stimulus. No account is taken of the reflex time itself. The sensory stimulus is the immersion of both the frog's feet once every minute into dilute HCl for not longer than 10 seconds, after which the feet are rinsed in water. The first solution used as stimulus is 0 05 N HCl, and when the frog fails to react, a stronger solution, 0.1 N HCl, is tested and after that 0.2 N HCl is tested. At this point stronger acid also

TABLE 2

Time taken for the development of plexus anaesthesia in frogs

CONCENTRATION OF COCAINE	FROG	TIME FOR ANAESTHESIA TO DILUTE HCl			MEAN TIME FOR ANAESTHESIA TO 0.2 N HCl
		0 05 N	0 1 N	0 2 N	
per cent		*min*	*min*	*min.*	
0.05	1	3	9	17	20.25 ±4.3 min.
	2	7	18	23	
	3	4	28	28	
	4	2	9	13	
0 1	5	4	6	8	9.7 ±1.0 min.
	6	4	8	11	
	7	3	4	12	
	8	5	7	8	
0.2	9	2	3	5	4.5 ±1.3 min.
	10	1	7	8	
	11	0	0	2	
	12	1	2	3	

fails to elicit withdrawal of the foot. The endpoint is determined in this way to avoid damaging the sensory nerve endings by repeated application of stronger acid than necessary. Three or four frogs can be tested simultaneously and for each the observation is made of the time taken by a given concentration of local anaesthetic to abolish the reaction to 0 2 N HCl. Table 2 illustrates an experiment carried out with three different concentrations of cocaine.

RESULTS. One of the two methods used determines the time taken for the anaesthesia to develop. The other determines the intensity and duration of anaesthesia. It was found that for all local anesthetics tested the same range of concentrations produced anaesthesia of the nerve plexus of the frogs and of the sensory endings in the skin of the guinea-pig.

For cocaine, however, there was a difference in the relation between concentration and "anaesthetic effect" in the two methods, i.e. doubling the concentration did not affect the time taken for anaesthesia to develop as much as it af-
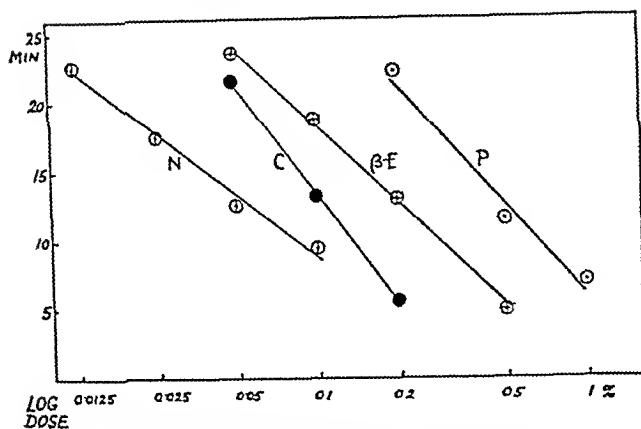
FIG. 1. PLEXUS ANAESTHESIA IN FROGS

The graph shows the relation between the concentration of the local anaesthetic (abscissae) and the time of onset of anaesthesia in minutes (ordinates). N = nupercaine, C = cocaine, β-E = β-eucaine, P = procaine. Each point represents the mean of 6–12 observations.
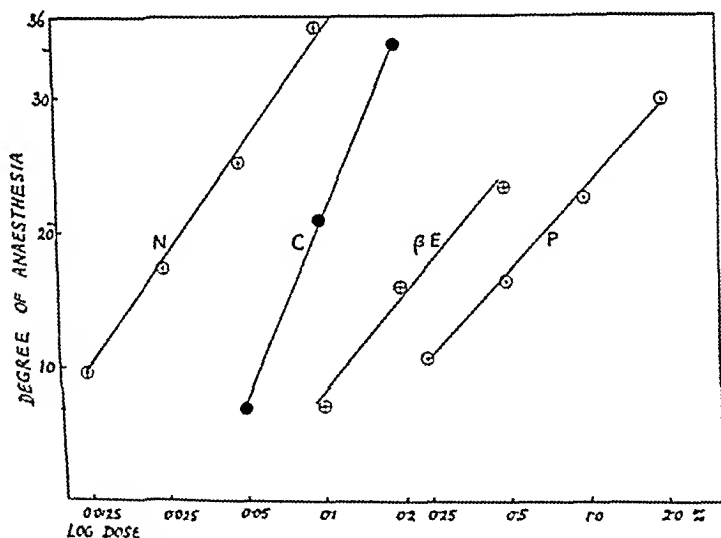
FIG. 2. INTRACUTANEOUS WHEAL IN GUINEA-PIGS

The graph shows the relation between the concentration of the local anaesthetic (abscissae) and the number of times there was no response to a prick; total 36 pricks in 30 minutes (ordinates). For nupercaine (N) each point represents the mean of 6 observations, for cocaine (C) of 33 to 90 observations, for β-eucaine (β-E) of 9–15 observations, for procaine (P) of 12–21 observations.

fected the duration and intensity of anaesthesia. If, as shown in figures 1 and 2, the concentration of cocaine is increased from 0.05% to 0.2% the time of onset of anaesthesia is reduced to nearly one-third (fig. 1), but the duration and intensity of anaesthesia is increased five times (fig. 2). The vasoconstrictor action of cocaine is probably responsible for this difference as the change in the effect produced by doubling the concentration was the same in the two methods for each of the other three anaesthetics.

In both methods there was a linear relation between the anaesthetic effect and the logarithm of the concentration of cocaine used. For the other three local anaesthetics tested the relation was also approximately linear but while the lines for these anaesthetics were parallel to one another, they were not paral-

TABLE 3

Potency of β-eucaine in terms of cocaine

(in brackets number of animals used)

METHOD	CONCENTRATION	EFFECT	% POTENCY (COCAINE = 100)
Guinea-pigs	0.05% Cocaine	3.3 (3) ±1.3	
	0.1% Cocaine	16.2 (6) ±1.3	
	0.2% Cocaine	33.3 (3) ±1.4	
	0 1% β-eucaine	4.0 (3) ±1.4	53.8%⎫ 51.3%
	0 2% β-eucaine	16 8 (6) ±1 5	48.9%⎭
Frogs	0.05% Cocaine	20.2 (4) ±3.3	
	0 1% Cocaine	9.5 (4) ±1.3	
	0.2% Cocaine	5 0 (6) ±0 9	
	0.1% β-eucaine	17 2 (4) ±1.4	54.9%⎫ 51.3%
	0.2% β-eucaine	11.5 (4) ±2.0	47.7%⎭

lel to the line for cocaine, the slope of which was steeper. Consequently the relative potency could not be expressed in terms of cocaine, since it varied according to the concentration used for the test. If low concentrations were chosen the potency appeared to be greater, and if high concentrations were chosen the potency appeared to be less in terms of cocaine. Thus tests were carried out in the following way. A determination of the effect of two or three different concentrations of cocaine was always made on the same day as that on which the compound was tested. Two doses were chosen of the latter which were likely to have effects intermediate between the effects of cocaine. By plotting the results obtained with cocaine against the logarithm of the concentration used the relative potency of each concentration of the tested compound was determined graphically. Thus several figures were obtained in one experiment and by taking the average an approximate value could be calculated. An example of a test is given in table 3 from which it is seen that both the guinea-pig and the frog method gave the same final result, that β-eucaine has 51.3 per cent of the potency of cocaine.

Procaine was found to be stronger when tested for plexus anaesthesia than by the intracutaneous wheal, whereas nupercaine was found to be stronger

when injected intracutaneously than when applied to the nerve plexus. The results obtained with the two methods are summarised in table 4. For the guinea-pig method the standard error of the mean was found to be about 10%

TABLE 4

Potency in terms of cocaine = 100

SUBSTANCE	INTRACUTANEOUS WHEAL (GUINEA-PIGS)					PLEXUS ANAESTHESIA (FROGS)				
	Lowest	Highest	Mean	Number of animals used for substance tested	Number of animals used for cocaine	Lowest	Highest	Mean	Number of animals used for substance tested	Number of animals used for cocaine
			per cent					*per cent*		
Procaine........	8.5	17.4	13.5	18	30	40.5	42.4	41.5	12	17
β-eucaine.	48.9	53.8	51.3	9	12	47.7	54.9	51.3	8	14
Nupercaine.....	255	315	282.5	12	12	140	224	169.5	14	14
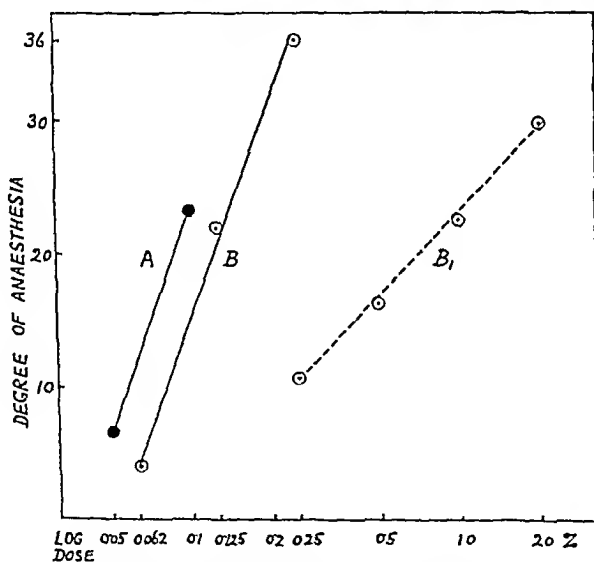
FIG. 3. INTRACUTANEOUS WHEAL IN GUINEA-PIGS

Abscissae = concentration of local anaesthetic, Ordinates = number of times there was no response to a prick; total 36 pin-pricks in 30 minutes. The slope of the line obtained for procaine (B′) becomes steeper when 1 in 100,000 adrenaline is added (B) and parallel to that for cocaine (A).

when 6 guinea-pigs were used for one concentration; it was about 13% for he frog method when 4 frogs were used for one concentration.

Procaine is usually given together with adrenaline. It was interesting to see how the addition of adrenaline affected the slope of the line relating the anaesthetic effect to the logarithm of the concentration of procaine. Figure

3 shows that without adrenaline the slope of the line of procaine is not so steep as that of cocaine, but when adrenaline 1 in 100,000 is added the line does not only shift towards the left (because lower concentrations become effective) but it also becomes parallel to the line of cocaine. In this experiment the potency of procaine in terms of cocaine was found to be 75% instead of 13.5% for procaine without adrenaline.

DISCUSSION. The two methods described follow well-known procedures, but they have been applied in such a way as to increase the accuracy. For this reason no determinations were made of threshold concentrations nor of maximal effects. The comparison was carried out in an intermediate range for which there is a linear relation between the logarithm of the concentration of the local anaesthetic and the anaesthetic effect in both the frog and the guinea-pig method. In the latter method, by applying six pricks every 5 minutes during a period of 30 minutes, an estimate of the degree of anaesthesia is obtained simultaneously with that of the duration. This index obtained from 36 pricks in 30 minutes was found to give a more accurate result than if the time was determined at which a 50% response, i.e. to 3 out of 6 pricks, occurred.

From figures 1 and 2 it is seen that while the lines relating the logarithm of the concentration to the anaesthetic effect of β-eucaine, procaine and nupercaine are not parallel to that of cocaine, they are parallel to each other. This suggests that it may be advisable to use procaine rather than cocaine as a standard for the assay of new compounds, but to add adrenaline when a comparison is made with cocaine, which itself has vasoconstrictor activity.

SUMMARY

1. Two methods are described for testing the potency of local anaesthetics.

2 With a certain range of concentrations a linear relationship exists between the logarithm of the concentration of the local anaesthetics which were examined and the time at which plexus anaesthesia in frogs develops.

3. When the intracutaneous wheal in guinea-pigs is used, a similar linear relation exists between the logarithm of the concentration of the local anaesthetic and an index expressing both intensity and duration of anaesthesia.

4. The lines for β-eucaine, procaine and nupercaine, though parallel to each other, are not parallel to that for cocaine. It is suggested, therefore, to use procaine as a standard of comparison. It was observed that when adrenaline was added to procaine and the mixture was compared with cocaine by the guinea-pig method, the log dose-effect lines became parallel.

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SYNTHETIC SUBSTITUTES FOR ATROPINE[1]

H. R. ING, G. S. DAWES AND IZABELLE WAJDA

From the Department of Pharmacology, Oxford

Atropine is an ester of an amino-alcohol (tropine) and α-phenyl β-hydroxy-propionic acid (tropic acid). Both halves of the molecule have been the subject of studies on the relation between structure and action. Jowett and Pyman (1) prepared a large number of tropine esters (tropeines) which were tested for mydriatic activity by Dale (see also Pyman, 2). The general conclusions were: that, for mydriatic activity on local application and in dilute solutions, the acidic moiety of the tropeine molecule must contain an aromatic nucleus; and that for a considerable degree of activity the esterifying acid must also contain an alcoholic hydroxyl group. No tropeine more active in man than atropine was discovered and the mandelic ester (homatropine) is the only one which has come into general use. It is curious that the readily available benzilic acid $(C_6H_5)_2 \cdot C(OH) \cdot CO_2H$ was not included in the acids investigated although it fulfils both the conditions mentioned above. Benzilyl-ψ-tropeine was investigated by Kreitmair (3) who observed that it had both mydriatic and anaesthetic effects on the eyes of rabbits and cats; ψ-atropine was stated by Liebermann and Limpach (4) to have no mydriatic action.

Attempts to modify the basic alcohol moiety of the atropine molecule have been less successful and only eucatropine (4-mandelyloxy-1:2:2:6-tetramethyl-piperidine), which is a feeble mydriatic, has been adopted in clinical practice. Von Braun, Braunsdorf and Räth (5), however, showed that the tropic esters of dialkylamino-ethanols and -propanols had mydriatic activity, the most active being the tropic ester of N-hydroxyethylpiperidine. Fromherz (6) reported that the tropic ester of γ-diethylamino-$\beta\beta$-dimethylpropanol (syntropan) had only feeble mydriatic activity, but that the corresponding quaternary ethobromide was more active; the benzilic ester and its methosulphate were however described as active mydriatics, the quaternary salt being again more powerful than the tertiary base. Halpern (7) reported that the benzilic ester of diethyl-aminoethanol was a mydriatic and local anaesthetic.

A more systematic study of the benzilic esters of simple alkamines was made by Blicke and Maxwell (8), who described the benzilic esters of hydroxyethyl-diethylmethylammonium bromide, hydroxyethyl-piperidine and its metho-bromide as "excellent mydriatics"; unfortunately no quantitative comparison with atropine was made. Later Blicke and Kaplan (9) provided evidence that simple alkamines yielded more active mydriatics when esterified with benzilic acid than with mandelic acid or any of the four possible phenylhydroxypropionic acids, including tropic acid.

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A more systematic study of the benzilic esters of simple alkamines was made by Blicke and Maxwell (8), who described the benzilic esters of hydroxyethyl-diethylmethylammonium bromide, hydroxyethyl-piperidine and its metho-bromide as "excellent mydriatics"; unfortunately no quantitative comparison with atropine was made. Later Blicke and Kaplan (9) provided evidence that simple alkamines yielded more active mydriatics when esterified with benzilic acid than with mandelic acid or any of the four possible phenylhydroxypropionic acids, including tropic acid.

Few of the synthetic mydriatics have been studied sufficiently closely for it to be certain that their activity is truly of the atropine type, i.e. due to antagonism of acetylcholine at the oculomotor endings and not to stimulation of the sympathetic mechanism in the eye. Dale made no attempt to settle this point when he tested the synthetic tropeines prepared by Jowett and Pyman and it may be noted that Blicke and Kaplan found that six of their benzilic esters, which were excellent mydriatics, were also excellent local anaesthetics on the rabbits cornea. At the same time the formal resemblance of these benzilic esters to esters of choline suggested to us that they might antagonize acetylcholine at cholinergic nerve endings in virtue of this structural similarity. Consequently the first compounds to be prepared and tested were the benzilic, tropic and atrolactinic esters of choline; all three esters were found to have mydriatic properties, the most active being benzilylcholine. Atrolactinylcholine had only feeble activity and tropylcholine was about half as active as benzilylcholine.

These results led to a systematic study of benzilic esters of the general formula: $(C_6H_5)_2 \cdot C(OH) \cdot CO \cdot O \cdot (CH_2)_n NR_1R_2R_3\}$ X where R_1, R_2 and R_3 are alkyl groups, X is an anion (Cl or Br), and n = 2 or 3. Later, benzilic esters analogous to eucatropine were also investigated. The compounds of both types which will be discussed below are listed in table 1, together with the serial numbers by which they will be designated in the text, the calculated molecular weights, the nature of the alkyl groups attached to the nitrogen atom and of the anion. All the compounds in table 1 were prepared in the Dyson Perrins Laboratory, Oxford, by one of us (H. R. I.) except E1, E2, E8, E10, E11, E14 and E15, which were prepared by Dr. A. H. Ford-Moore; compounds E1, P1 and C4 and bromides corresponding to E2 and P2 have been already described by Blicke and his collaborators. 4-Hydroxy-1:2:2:6-tetramethylpiperidine exists in two stereoisomeric forms, α- and β-, eucatropine being the mandelic ester of the β-form; P8, P9 and P10 are derived from the β-form and P11 and P12 from the α-form.

In order to discover whether these synthetic mydriatics resembled atropine in tissues other than the eye, a study of their action on the salivary gland of the cat has been made; a few compounds have also been tested on isolated rabbit's intestine and on the isolated cat's heart (Langendorff's preparation).

The observation that among our compounds quaternary metho-salts were invariably more active than the corresponding tertiary bases has led us to re-examine the action of the metho-salts of atropine, l-hyoscyamine, l-hyoscine and eucatropine on the eye; Bülbring and Dawes have already described their actions on the salivary gland (10).

BIOLOGICAL OBSERVATIONS *Tests for mydriatic activity.* It is not commonly known that a simple and relatively accurate test for mydriatic activity has been described by Pulewka (11). The method requires mice and no other apparatus than a binocular microscope magnifying about ten times and provided with a scale in the eyepiece with which to examine and measure the diameter of the pupil of the mouse. A strong light shining into the eye of the mouse must be attached to the microscope.

TABLE 1

SERIAL NUMBER	Name	Molar weight	R₁	R₂	R₃	Anion
			$(C_6H_5)_2 \cdot C(OH)CO\ O\ CH_2\ CH_2\ N[X$ with $R_1, R_2, R_3]$			
C4	Benzilyloxyethyl-dimethylammonium chloride	335.5	CH_3	CH_3	H	Cl
C1	Benzilylcholine chloride ($+\frac{1}{2}$ H_2O)	358.5	CH_3	CH_3	CH_3	Cl
E3	Benzilyloxyethyl-dimethylethylammonium chloride	363.5	CH_3	CH_3	C_2H_5	Cl
E4	Benzilyloxyethyl-dimethylisopropyl-ammonium chloride	377.5	CH_3	CH_3	$CH(CH_3)_2$	Cl
E5	Benzilyloxyethyl-dimethyl-n-propyl-ammonium bromide	422	CH_3	CH_3	C_3H_7	Br
E6	Benzilyloxyethyl-dimethylallylammonium bromide	420	CH_3	CH_3	C_3H_6	Br
E7	Benzilyloxyethyl-dimethyl-n-butyl-ammonium bromide	436	CH_3	CH_3	C_4H_9	Br
E11	Benzilyloxyethyl-dimethyl-n-amyl-ammonium bromide	450	CH_3	CH_3	C_5H_{11}	Br
E1	Benzilyloxyethyl-diethylammonium chloride	363.5	C_2H_5	C_2H_5	H	Cl
E2	Benzilyloxyethyl-diethylmethylammonium chloride	377.5	C_2H_5	C_2H_5	CH_3	Cl
E14	Benzilyloxyethyl-triethylammonium bromide	436	C_2H_5	C_2H_5	C_2H_5	Br
E15	Benzilyloxyethyl-diethyl-n-propyl-ammonium bromide	450	C_2H_5	C_2H_5	C_3H_7	Br
P1	Benzilyloxyethyl-piperidine hydrochloride	375.5	$\cdot CH_2(CH_2)_3 \cdot CH_2 \cdot$		H	Cl
P2	Benzilyloxyethyl-piperidine metho-chloride	389.5	$\cdot CH_2(CH_2)_3 \cdot CH_2 \cdot$		CH_3	Cl

SERIAL NUMBER	NAME	Molar weight	R₁	R₂	R₃	Anion
			$(C_6H_5)_2\ C(OH)CO\ O\ CH_2\ CH_2\ CH_2\ N[X$ with $R_1, R_2, R_3]$			
E8	Benzilyloxypropyl-diethylmethylammonium chloride	391.5	C_2H_5	C_2H_5	CH_3	Cl
E10	Benzilyloxypropyl-dimethylethylammonium chloride	377.5	C_2H_5	CH_3	CH_3	Cl

SERIAL NUMBERS	Name	Molar weight	R₁	R₂	Anion
			$(C_6H_5)_2\ C(OH)CO\ O\ CH$ with $CH_2\!-\!CMe_2 / CH_2\!-\!CHMe$ $N[X$ with $R_1, R_2]$		
P8	4-Benzilyloxy-1,2,2,6-tetramethylpiperidine hydrochloride (β)	403.5	CH_3	H	Cl
P9	4-Benzilyloxy-1,2,2,6-tetramethylpiperidine methoiodide (β)	509	CH_3	CH_3	I
P10	4-Benzilyloxy-1,2,2,6-tetramethylpiperidine methochloride (β)	477.5*	CH_3	CH_3	Cl
P11	4-Benzilyloxy-1,2,2,6-tetramethylpiperidine methoiodide (α)	509	CH_3	CH_3	I
P12	4-Benzilyloxy-1,2,2,6-tetramethylpiperidine methochloride (α)	477.5*	CH_3	CH_3	Cl

* P10 and P12 crystallise from isopropanol with one molecule of isopropanol of crystallization.

Groups of mice are taken, preferably from 15–20 g. in weight; larger mice are less sensitive, though this does not preclude their use, provided that all those used in one experiment are similar in weight. They are not kept without food beforehand. Pulewka recommended using mice of one sex, but this has not been found necessary. The mice are injected intraperitoneally with 0.2 cc. of the solution to be tested and the effect on the pupil is determined. The action of a dose of atropine reaches its maximum in 15 min. and starts declining after 25 min.; for this reason readings were taken within 15–20 min. after the injection.

The diameter of the pupil in uninjected mice was found to vary between 1 5 and 2 5 divisions of the scale in the microscope eyepiece (7.7 divisions = 1 mm.); this diameter was unaffected by excitement. After the injection of 0.002 mg. atropine sulphate the aver-
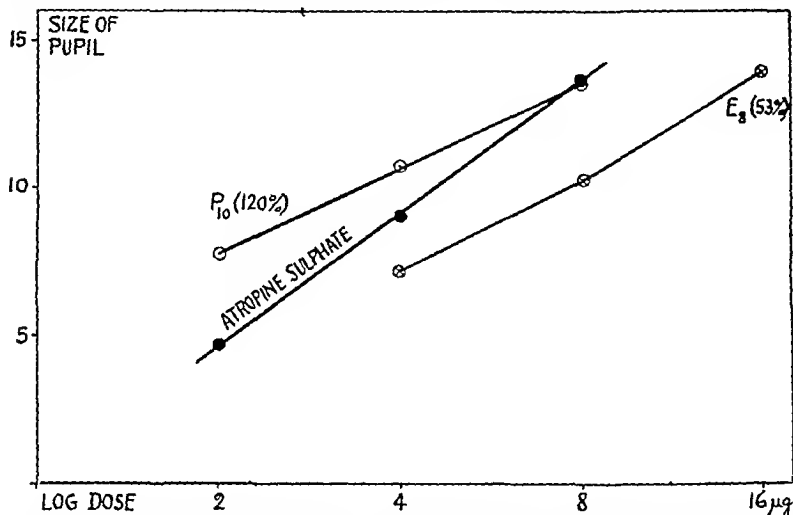
FIG. 1. LOG-DOSE—ACTION CURVES FOR ATROPINE AND SYNTHETIC MYDRIATICS
Ordinates· Diameter of mouse pupil in divisions of microscope scale. Abscissae.
Logarithm of dose in micrograms.

age diameter was usually about 4 divisions, while after 0 008 mg , the diameter was about 14 divisions; the maximum diameter recorded was 18 divisions. The interesting observation was made that when doses of atropine sulphate were given in the range 0.002–0 008 mg., the mean diameter of the pupil in similar groups of mice was directly proportional to the logarithm of the dose This is shown in figure 1, in which the points were obtained by combining ten experiments, each point representing the mean of results in 100 mice.

The effect of a given dose of atropine sulphate varied in different groups of mice, and even within the same group at different times For this reason, in comparing a substance of unknown potency with atropine, the effect of two or three different doses of atropine sulphate in different groups of mice was always determined simultaneously with that of a range of doses of the substance under comparison. By plotting the results obtained with atropine as in figure 1, the effect of a dose of the unknown could be equated to that of a dose of atropine sulphate.

In practice it was found convenient to use 5 mice in a group, and to inject three groups. The diameters of the pupils were then determined. The experiment was continued by injecting 3 more groups and so on. The details of part of an experiment in which l-hyoscine hydrobromide was compared with atropine sulphate are given in table 2. The part

illustrated occupied one worker about one hour, excluding the time taken to prepare the solutions; the complete experiment involved repeating the same doses twice on fresh groups of mice so that the effect of each dose of each substance was observed in 15 mice. Mice

TABLE 2

SUBSTANCE	DOSE PER MOUSE	TIME OF		PUPIL DIAMETERS	MEAN
		Injection	Examination		
	$m\varrho$				
Atropine Sulphate	0.002	11.18	11.33	2, 3, 2, 7, 5	3.8
	0.004	11.21	11.36	4, 6, 9, 2, 11	6.4
	0.008	11 24	11.39	15, 15, 15, 4, 17	13.2
Hyoscine Hydrobromide	0.00025	11.51	12.06	4, 2, 2, 3, 5	3 2
	0.0005	11.55	12.10	7, 4, 6, 7, 6	6.0
	0.001	11 58	12.13	12, 12, 14, 12, 8	11.6

TABLE 3

SUBSTANCE	POTENCY RELATIVE TO ATROPINE SULPHATE = 100	RELATIVE POTENCY CALCULATED PER MOL ATROPINE = 100	NUMBER OF MICE USED	
			for substance	for atropine
C4	13	12 6	15	15
C1	30	31	50	65
E3	100	104 4	80	90
E4	85	92 5	60	70
E5	18	22	24	20
E6	23	28	20	20
E7	8	10 5	25	20
E11	10	13	28	35
E1	6	6 3	25	50
E2	59	64	55	55
E14	66	83	85	85
E15	17	22	60	85
P1	0 5		15	40
P2	16	17 3	15	40
E8	53	60	55	80
E10	59	64	50	80
P8	14	16	30	40
P9	116	170	60	60
P10	120	165 4	65	50
P11	133 5	196	20	30
P12	104	143	40	75

were not used more often than twice in one week, and after three to four weeks' use they were discarded because they grew bigger and less sensitive.

Results. The results obtained with the series of benzilic esters are shown in table 3 in which the substances have been arranged not in the consecutive order of their serial numbers, but in the order of the homologous series as in

table 1. The numbers of mice used in each comparison for atropine and for the synthetic substances are shown in Columns 4 and 5. If a substance appeared to have little mydriatic action compared with atropine, only a few mice were used. Seven of the compounds listed in table 3 proved to be highly active mydriatics: E3 was equal to atropine sulphate and E4 had 85 per cent of the activity of atropine sulphate; on a weight basis E14 had 66 per cent of the activity of atropine sulphate, but because of its higher molar weight it has about 83 per cent of the activity of atropine on a molar basis. P9, P10, P11 and P12 were all more active than atropine sulphate. P9 and P11 are the iodides of two stereoisomeric bases; P10 and P12 are the corresponding and more soluble chlorides. On a molar basis P9 and P10 should have equal potencies since they are salts of the same base; reference to the table will show that their molar potencies differ by less than 3 per cent. For the same reason the molar potencies of P11 and P12 should also be equal but owing to its low solubility in water P11 was tested on too few mice for the figure quoted in the table to be reliable.

The potencies listed in table 3 are average figures. The curves relating log. dose to effect for the synthetic mydriatics are frequently not parallel to that of atropine (fig. 1); the curve for E3 coincides with that for atropine, but that for P10 converges with, and that for E8 diverges from, the curve for atropine as the dosage is increased. When the log. dose—effect curve of a substance is not parallel to that of atropine it is impossible to give a relative potency figure which will be true at all doses and an average figure for a four or fivefold dosage range is given.

Duration of mydriasis. The potencies shown in table 3 are those obtained 15 min. after injection. The duration of mydriasis is also important since one of the most characteristic and valuable properties of atropine is the prolonged effect which it produces in the eye. None of the synthetic substances had so prolonged an action as atropine. In figure 2 the mean size of the mouse pupil, measured at 30 min. intevals, is plotted as a multiple of its normal size against time both for atropine and for some of the more active synthetic substances. It will be noticed that the effect of E3 was sustained better than that of the other substances; the effect of P10 and P12, though initially greater than that of E3, passed off very quickly.

The duration of mydriasis produced by E3 and E14 was also investigated by local administration of one drop of one per cent solutions twice daily into the eyes of cats. In cats atropine maintains full dilatation of the pupil during five days of such treatment and for five days after administration has ceased. E3 showed little cumulative effect, some constriction to light occurring each morning during the treatment; complete recovery was observed 24 hours after administration had ceased. E14 had a more cumulative effect than E3 while the treatment was continued but its action had also disappeared 24 hours after administration had ceased.

Action on the salivary gland. The synthetic benzilic esters were found to resemble atropine not only in the eye but also in antagonizing the salivary sc-
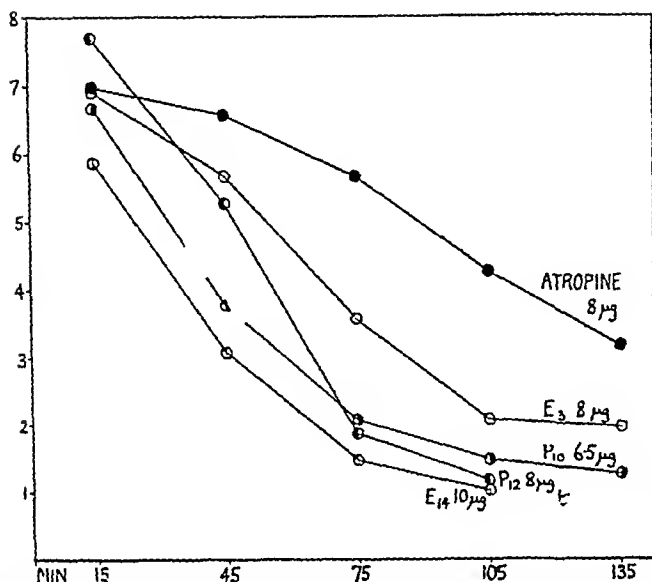
FIG. 2. ACTION—DURATION CURVES FOR ATROPINE AND SYNTHETIC MYDRIATICS
Ordinates: Diameter of mouse pupil expressed as a multiple of normal diameter. Abscissae: Time in minutes.

TABLE 4

SERIAL NUMBER OF SUBSTANCE	RELATIVE POTENCIES PER MOLECULE IN TERMS OF ATROPINE = 100		
	On the salivary gland (cat)	On the blood pressure (cat)	On the eye (mouse)
C4	11.0 ±1.8	9.8	12.6
C1	196 ±32	103	31
E3	258 ±38	182	104.4
E4	293 ±59	238	92.5
E5	147 ±46	98	22
E7	75.4 ±19	63	10.5
E11	71.3 ±36	32	13
E1	17.8 ±4.6	21	6.3
E2	273 ±49	239	64
E14	273 ±44	190	83
E15	135 ±30	78	22
P1	4 ±1.4	3	—
P2	185 ±66	70	17.3
P8	14.6 ±1.75	14.6	16
P9	152 ±78	103	170

cretion produced by pilocarpine and carbaminoyl choline.   Fifteen of them were
compared with atropine on the salivary gland of the cat.   The method used has
been described by Bülbring and Dawes (10).   Each substance was tested on
not less than five cats. ·The results are recorded in table 4 in which the rela-
tive potencies per molecule on the salivary gland and on the blood pressure are
expressed in terms of atropine = 100; beside each figure for the relative molar
potency on the salivary gland is given the standard deviation.   The relative
molar potencies on the eye are included in the table in order to facilitate com-
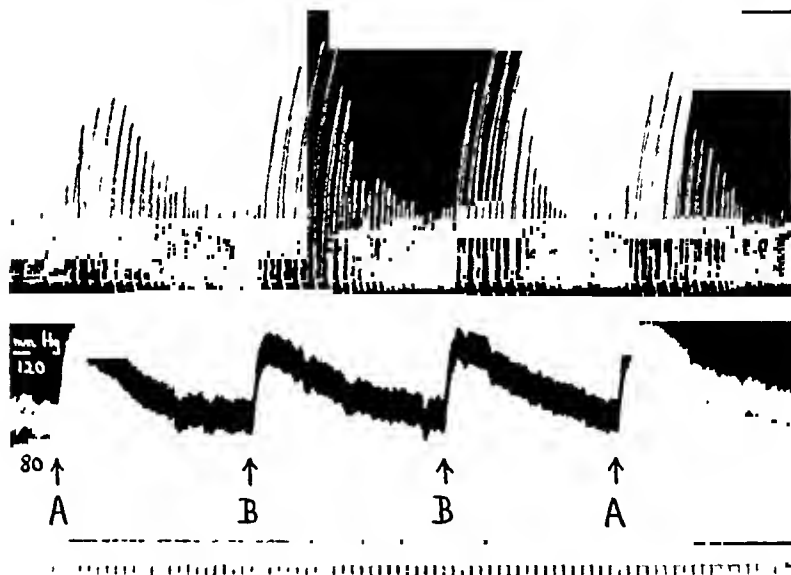
FIG. 3. CAT, 2 6 KG. PENTOBARBITONE. INFUSION OF CARBACHOL, 1:25,000 AND ADREN-
ALINE, 1:50,000

Above: salivary flow, recorded by Gaddum's drop-recorder. Below blood pressure.
At A, 3 µg. atropine sulphate, at B, 1 µg E3

parison. One record obtained in comparing E3 with atropine is illustrated
in figure 3.

Inspection of table 4 will show that the synthetic substances have a more
powerful effect on the salivary gland than on the blood pressure and that sev-
eral of them are considerably more potent than atropine on both. The my-
driatic potencies in the mouse are generally less than the potencies on the
salivary gland and on the blood pressure of the cat; P9 constitutes the only
reliable exception to this observation, the irregularities shown by the salts of
tertiary bases (C4, E1, P1, and P8) being of doubtful significance in view of the
feeble potencies of these substances In general the substances which are most
active on the pupil of the mouse are among the most active on the salivary

gland and on the blood pressure of the cat; similarly the weakest mydriatics exert also the weakest actions on the salivary gland and on the blood pressure. A few substances however, notably C1, E2 and P2, which are less active than atropine on the pupil of the mouse are considerably more active than atropine on the salivary gland of the cat.

Action on smooth muscle. Three of the synthetic substances, viz., E3, E10 and E14, were tested for inhibitor action on rabbit intestine and compared with atropine. A regular series of contractions of an isolated piece of rabbit intestine was obtained by adding constant doses of acetylcholine at constant time intervals to the bath in which it was suspended. Two doses of atropine were found which produced temporary reductions of the contractions and the percentage reductions produced by E3, E10 and E14 were compared with those produced by atropine. The relative potencies, calculated in terms of atropine = 100, were: E3 = 104, E10 = 55 and E14 = 87. These figures are very close to the relative potencies of these substances as mydriatics (see table 3).

TABLE 5

LD50 for mice (mg. per 20 g.)

SUBSTANCE	INTRAPERITONEALLY	SUBCUTANEOUSLY	ORALLY
E2	1.25	2.6	
E3	0.8	3.2	20.0
E4	0.8	1.5	
E8	1.8	11.0	
E10		13.0	
E14		3.0	
P10	1.5	6.5	20.0
P12	1.6	7.5	
Atropine	6.4	15.0	15.0

Action on the heart. E3 was found to antagonize acetylcholine in the isolated perfused cat's heart (Langendorff's preparation). The inhibition produced by acetylcholine in this preparation is abolished or reduced by doses of atropine ranging from 0.02 to 1 microgram. The reductions produced by E3 and atropine in equal doses were found to be identical, but the effect of E3 was shorter, being about half as long as that of atropine.

Toxicities. Eight of the more active mydriatics among the synthetic compounds were tested for toxicity on mice and the results are recorded in table 5. All of them proved to be more toxic than atropine on intraperitoneal or subcutaneous injection. Two of the most promising compounds E3 and P10, were also tested by oral administration and were found to be less toxic than atropine by this route.

No figures for the lethal doses in cats have been obtained, but the toxic symptoms produced by atropine have been compared with those produced by E3 and P10. In a cat of 2.3 kg. 138 mg. atropine (i.e. 60 mg. per kg.) given subcutaneously produced severe tremor, rapid respiration (120 per min.) and

vomiting. Gradually a state of severe excitement developed and the cat had several fits of violent convulsions. After 6 hours the convulsions stopped, the animal appeared exhausted, its legs were spastic and it could not stand up. However, the cat recovered and showed no symptoms except maximal mydriasis on the following day.

The symptoms produced by the same dose of E3 were different. A cat of 2.0 kg. received 120 mg. E3 (i.e. 60 mg. per kg.) subcutaneously; during the first 15 min. slight tremor appeared, the respiration quickened to 65 per min., and the cat gradually went to sleep. When aroused it appeared confused and could walk only with difficulty. After 2 hours it began to wake up and after 5 hours the cat could walk though it was still sleepy. Next day no symptom except mydriasis was detected.

In another cat 30 mg. E3 per kg. produced some sleepiness, slight ataxia but, except for mydriasis, no other symptoms.

P10 was injected into three cats in doses of 30 mg., 60 mg. and 120 mg. per kg. The small dose produced no appreciable effect. The middle dose produced ataxia, unsteadiness and incoordination in walking. The large dose made the cat sleepy, but also produced vomiting and severe ataxia.

Thus E3 and P10 were found to have an action on the cat more like that typical of scopolamine. The symptoms produced by atropine given in the same dose were more severe than those of E3 or P10.

The metho-salts of atropine, hyoscyamine and hyoscine. Crum Brown and Fraser (12) were the first to examine the pharmacology of atropine methiodide and methosulphate. They found that both metho-salts retained the mydriatic action of atropine undiminished; the action on the vagi was also retained but the effects on the C.N.S. were absent; both salts exhibited a much more powerful curare-like action than atropine. Atropine methonitrate (eumydrine) was examined clinically in the early years of this century and was reported to be considerably weaker and shorter in its mydriatic action than atropine (cf. Duke-Elder, 13).

Issekutz (14) estimated atropine methobromide to be eight times as strong as atropine on the frog's vagus, 3–4 times as strong as atropine on the salivary gland of the rabbit, and about equal to atropine on the pupil. Cushny (15) concluded that atropine methobromide was about fifty per cent stronger than atropine in antagonizing pilocarpine in the salivary gland of the dog. More recently Nyman (16, 17) has compared the metho-salts of atropine and l-hyoscine with the parent alkaloids; he found that atropine methonitrate was about twice as active as atropine sulphate in antagonizing the salivary secretion produced by pilocarpine in man, but that l-hyoscine methonitrate and methobromides were respectively 20 per cent more and 20 per cent less active than l-hyoscine in the same property. In the human eye the metho salts of l-hyoscine were about equal in mydriatic activity to l-hyoscine, but atropine methonitrate was about 25 per cent more active than atropine.

We have compared the actions of atropine methonitrate, l-hyoscyamine and its methiodide, l-hyoscine and its methiodide and eucatropine and its methiodide

with atropine on the eye of the mouse and of the cat by methods already used for the synthetic substances. The results are collected in table 6.

Bülbring and Dawes (10) found that the metho-salts of atropine, l-hyoscyamine and l-hyoscine were each about twice as active as their parent alkaloids in the salivary gland and on the blood pressure of the cat. The relative mydriatic activities of these pairs of tertiary and quaternary bases depend upon the method of administration; thus atropine and l-hyoscyamine sulphate are more active than their respective methiodides when instilled into the conjunctiva sac of the cat, but less active than the methiodides when injected into the peritoneal cavity of the mouse. l-Hyoscine hydrobromide and l-hyoscine methiodide differ from the two other belladonna alkaloids in this respect; they appear to be about equally active in the eye of the mouse after intraperitoneal

TABLE 6

SUBSTANCE	RELATIVE POTENCIES PER MOLECULE (ATROPINE = 100) ON THE EYE OF THE:			
	Mouse, intra-peritoneally	Number of mice	Cat, locally	Number of cats
Atropine sulphate .	100		100	
Atropine methonitrate	228	30	50–100	9
l-Hyoscyamine sulphate .	185	85	200	2
l-Hyoscyamine methiodide	492	60	100	2
l-Hyoscine hydrobromide	491	75	1000–1500	9
l-Hyoscine methiodide	479	35	330	3
Eucatropine hydrochloride	0.4	30	0.05	7
Eucatropine methiodide	2.8	30	0.04	2

injection, but l-hyoscine hydrobromide is about four times as active as the methiodide when both are applied locally to the eye of the cat.

When l-hyoscyamine and l-hyoscine were compared directly with the corresponding methiodides by local application to the eyes of a cat, l-hyoscyamine was found to be 2–4 times as powerful as its methiodide (10 cats) and l-hyoscine 3 times as powerful as its methiodide (2 cats).

The difference in the relative mydriatic activities in the mouse and in the cat is more probably caused by the different methods of administration than by the difference in species. The relative weakness of the methiodides on local application may be due to relatively poor absorption from the conjunctival sac. In order to throw some light on this point and also on the apparently anomalous behaviour of l-hyoscine and its methiodide in the mouse, several experiments were carried out on cats under pentobarbitone anaesthesia. The pupil was kept constricted by a constant infusion of carbaminoylcholine and adrenaline as in the salivary secretion experiments. The method was tedious because large intravenous doses of atropine (60 μg.) were required to dilate the

pupil whereas 1–5 μg. atropine was sufficient to stop the salivary flow. However when l-hyoscine hydrobromide and l-hyoscine methiodide were compared with atropine in this preparation, l-hyoscine proved to be less potent than its methiodide: l-hyoscine was about equal to atropine and l-hyoscine methiodide about 60 per cent more active. Neither substance was so powerful under these conditions as when it was applied locally, but the experiment demonstrates that the ratio of the activities of these two drugs is dependent upon the method of administration.

The apparently anomalous behaviour of l-hyoscine and its methiodide in the mouse is also probably related to some such factor as rate of absorption at the
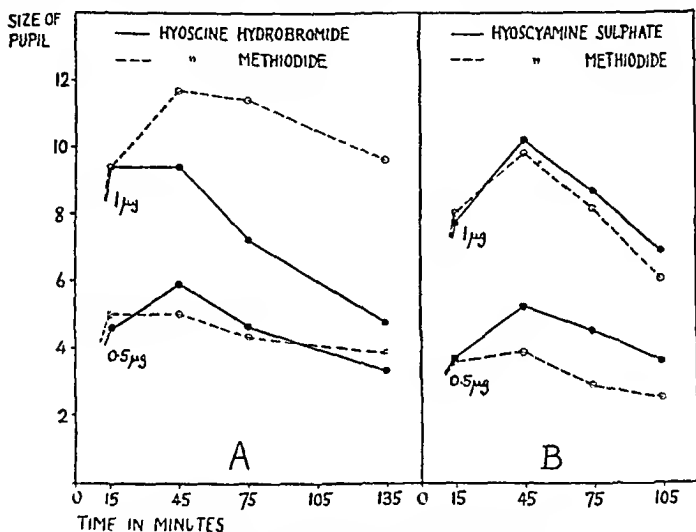
FIG. 4. ACTION—TIME CURVES FOR MOUSE PUPIL

Ordinates: Diameter of pupil in divisions of microscope scale. Abscissae: Time in minutes.

Continuous curves: A. l-Hyoscine hydrobromide; B. l-Hyoscyamine sulphate. Interrupted curves: A. l-Hyoscine methiodide; B. l-Hyoscyamine methiodide.

site of action. Thus when the potencies were estimated 15 min. after intraperitoneal injection they were approximately equal, as recorded in table 6, but the subsequent course of the mydriasis depended on the dose (fig. 4A). In 1 μg. doses the methiodide achieved its maximum dilatation later than hyoscine and was more powerful than the latter; in 0.5 μg. doses the methiodide was not more powerful than hyoscine but its action declined more slowly. The apparent intensity of a drug action will depend not only upon the intrinsic intensity but also upon the balance achieved between rates of penetration to and disappearance from the site of action. The curves in figure 4A suggest that the methiodide accumulates at the site of action more slowly than l-hyoscine. This behaviour is, however, not typical of quaternary salts; thus the time course o

mydriasis in the mouse with l-hyoscyamine methiodide remains parallel with that of l-hyoscyamine sulphate over 105 min. (fig. 4B).

The results with eucatropine hydrochloride and its methiodide (table 6) are chiefly of interest because these substances are esters of mandelic acid and P8 and P9 are the analogous esters of benzilic acid; the substitution of a phenyl group for the α-H-atom of the mandelyl group increases the mydriatic activity in the mouse some 40 to 60 times.

DISCUSSION. Of the seventeen benzilic esters of quaternary alkamines listed in table 1, eleven have been tested not only on the eye of the mouse but also on the salivary gland and on the blood pressure of the cat; in every case a qualitative resemblance to atropine was observed. E3 also resembled atropine closely in smooth muscle and in the isolated cat's heart, and E10 and E14 antagonized acetylcholine in smooth muscle. The compounds differed quantitatively from atropine both in the intensity and in the duration of their effects, but whereas several of them were markedly more powerful than atropine, none of them produced so prolonged an action on the pupil.

The results provide strong presumptive evidence in favour of the view that these quaternary salts are truly atropine-like and owe their pharmacological properties to an antagonism of acetylcholine. This conclusion is of considerable theoretical interest because all the E-compounds are benzilic esters of quaternary alkamines analogous to choline and C1 is the benzilic ester of choline itself. The only other instance of an atropine-like ester of choline known to us is di-n-butylcarbamylcholine, recently investigated by Swan and White (18), but some doubt exists about the exact site and mechanism of its action. Choline esters with nicotine or curare-like properties have been known for many years, e.g. the nitrous ester (19), but the benzilic and dibutylcarbamic esters appear to be the first examples of atropine-like choline esters.

In considering the relations between chemical structure and pharmacological action among our compounds, three structural features call for mention: the nature of the esterifying acid, the size of the alkyl groups and the state of the nitrogen atom (whether tertiary or quaternary).

The compounds in table 1 all contain the benzilic acid radical and consequently fulfil the conditions observed by Jowett and Pyman (1) to hold for marked mydriatic activity among tropeines, viz. an aromatic nucleus and an alcoholic hydroxyl group; they do not contain an asymmetric centre which some authors have regarded as essential for a high degree of mydriatic activity. Blicke and Kaplan (9) found that alkamine esters of benzilic acid were in general more likely to be good mydriatics than similar esters of mandelic acid or any of the four phenylhydroxypropionic acids; in agreement with this we found that tropylcholine was less active than benzilylcholine and the replacement of mandelic by benzilic acid in eucatropine and its methiodide increased the mydriatic activity some forty to sixtyfold; thus the molar potencies for these four compounds are: eucatropine hydrochloride 0.4 and P8 16; eucatropine methiodide 2 8 and P9 170 (atropine = 100)

The effect of changing the alkyl groups in our compounds is best appreci-

ated by consideration of figure 5, in which the results for two homologous series
are represented graphically. It will be noticed that changes in the size of one
alkyl group which lead to an increase or to a decrease in mydriatic activity lead
to similar changes in the two other atropine-like activities. The members of
each homologous series are in general more potent on the salivary gland than
on the blood pressure and more potent on the blood pressure than on the pupil.
The maximum activity in the alkyl-dimethyl ammonium series is sharply de-
fined for the ethyl-dimethyl member, but in the alkyl-diethyl ammonium series
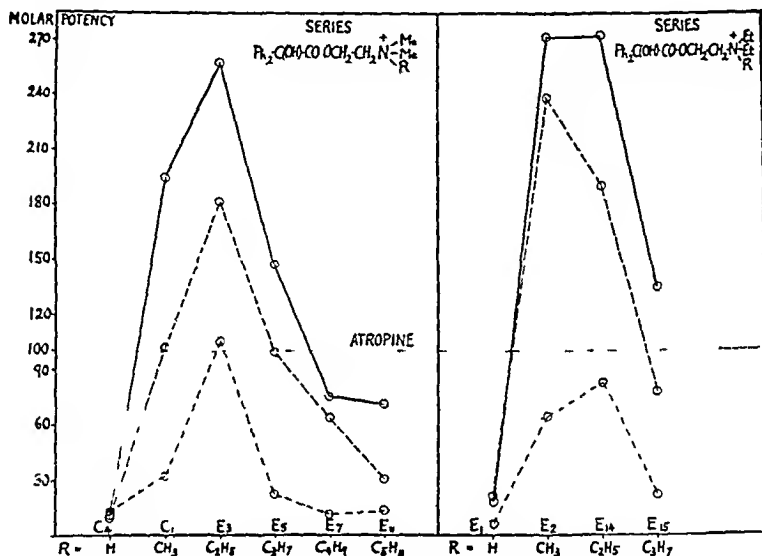
FIG. 5. POTENCY VARIATIONS IN HOMOLOGOUS SERIES

Ordinates: Molar potency in terms of atropine = 100. Abscissae Number of carbon
atoms in alkyl radical R.

Potencies on the salivary gland (continuous lines), on blood pressure (broken lines)
and on the mouse pupil (dot and dash lines)

the maximum is less well defined, E2 being more active on the blood pressure
and less active on the pupil than E14, but equal to it in the salivary gland.

With regard to compounds not represented in figure 5, it may be noted that
isopropyl was a more effective group than n-propyl in the alkyl-dimethylam-
monium series (i.e. E4 more active than E5) and that the n-propyl (E5) and
allyl (E6) members were about equally active. Both P1 and the bromide
corresponding to P2 were reported by Blicke and Maxwell (8) to be excellent
mydriatics on the rabbit's cornea; in our tests these two compounds were rela-
tively feeble mydriatics. Lengthening of the carbon chain between the nitro-
gen atom and the ester group from 2 to 3 carbon atoms (E8 and E10) decreased

the toxicity (table 5) but did not increase the mydriatic activity; thus E8 was about equal in activity to its analogue E2, but E10 was less active than its analogue E3.

The striking increase in activity in passing from the salt of a tertiary base to the quaternary metho-salt is well illustrated in figure 5; both C4 and E1 are only feebly active in all three properties, but C1 and E2 are relatively powerful atropine-like drugs. A similar increase in activity was observed in passing from the tertiary bases P1 and P8 to the corresponding quaternary metho salts P2 and P10. The four salts of tertiary bases, C4, E1, P1 and P8 are also local anaesthetics and it is hoped to publish details of their activity in this respect later. Meanwhile some doubt must remain whether they are truly atropine-like in their action. E1 has already been shown to possess local anaesthetic and antispasmodic properties (20, 21).

The metho-salts of the natural belladonna alkaloids also appear to be more active than their parent bases. Bülbring and Dawes (10) have confirmed and extended the work of previous authors on the salivary gland and on the blood pressure, using the cat; the metho-salts were uniformly more active than the tertiary bases. We have found that the relative potencies of the belladonna alkaloids and their metho-salts in the eye depend upon the method of administration. Applied locally to the eye of the cat the natural alkaloids are more potent than their metho-salts, but in man Nyman (16, 17) found atropine metho nitrate slightly more active than atropine sulphate and the metho-salts of l-hyoscine about equal in activity to l-hyoscine hydrobromide. Injected intraperitoneally in the mouse the metho-salts of atropine and l-hyoscyamine were more powerful mydriatics than their parent bases, but l-hyoscine and its methiodide were about equal. Eucatropine methiodide was also more active in the eye of the mouse than eucatropine hydrochloride and a similar relation held for the salivary gland (11). Finally Nyman (16) found homatropine metho nitrate more active than homatropine in the salivary gland and in the eye of man.

The general conclusion appears to be that the quaternary metho-salts of all these compounds are intrinsically more active than the corresponding tertiary bases, but that in the eye the metho-salts may appear less active when they are applied locally. Local application to the eye is undoubtedly the most useful test of mydriatic activity for clinical purposes, but as a test of intrinsic potency it is less satisfactory because it involves such factors as the absorption of the drug from the conjunctival sac and its penetration to the site of action. The lower activity of the metho-salts on local application is not surprising because it is well-known that onium cations penetrate cell membranes much more slowly than tertiary bases, the latter existing in solution not only as the cation of a salt but also as the free base. l-Hyoscine and its methiodide present a genuine anomaly, but these drugs contain a functional group, the oxide ring, absent from all other mydriatics, which may modify its properties.

The superior activity of metho-salts to that of tertiary bases among both the atropine alkaloids and our synthetic atropine substitutes has interesting impli-

cations.   All these drugs belong to the same structural type; they are alkamine esters of the general formula:

$$\text{Acyl-O-(C)}_n\text{-}\overset{+}{\text{N}}\overset{\displaystyle R_1}{\underset{\displaystyle R_3}{\diagdown}}\text{R}_2 \Big\} \overset{-}{\text{X}}$$
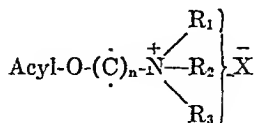
where the $(C)_n$ group is either a polymethylene chain as in the E-compounds or a cyclic structure as in the belladonna alkaloids and the P8 to P12 compounds. It is a striking fact that compounds of this general type have a peculiar affinity for neural structures and exhibit one or more of four kinds of pharmacological action, viz. cholinergic, atropine-like, curare-like and local anaesthetic, depending on the nature of the acyl radical and of the basic group.

Cholinergic properties appear to be rather strictly confined to molecules containing 3 methyl groups on the N-atom and an aliphatic acyl radical (acetyl, pyruvyl, carbamyl, etc.); the $(C)_n$ group is usually $(CH_2)_2$ but n may be 3 or the chain branched, e.g. acetyl β-methylcholine.

Atropine and curare both antagonise acetylcholine drugs but the anatomical distribution of their effects is different.   Curare-like properties are widely shared by alkamine esters provided that the basic group is quaternary, but they may appear in a milder and more transient form in tertiary bases.   The curare type of activity is characteristic of the onium cation and when it is displayed by tertiary bases most probably depends upon a relatively stable tertiary cation $R_3\overset{+}{N}H$, because strong bases show it more markedly than weak bases (22).

The results, which are reported in this paper, suggest that atropine-like properties ought also to be attributed to the cation and not to the free base. Conversion of a tertiary base into its metho-salt stabilizes the cation.   Whereas tertiary cations $R_3\overset{+}{N}H$ exist in equilibrium with the free base, thus:

$$R_3\overset{+}{N}H + \overset{-}{O}H \rightleftharpoons R_3N + H_2O$$
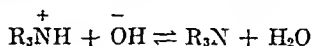
quaternary cations are incapable of such a reversible reaction.   We have found that such stabilization normally increases the atropine-like properties of tertiary bases.   The attribution of atropine-like properties to the cation of the tertiary base not only explains our results but also brings the atropine-like action into line with the curare-like action; both are due to antagonism of an onium cation, acetylcholine, in cells adapted to respond to the latter, and our view is that the cationic nature of the antagonist, whether atropine-like or curare-like, is fundamentally important.   The conversion of a tertiary base with atropine-like properties into its metho-salt not only increases its activity in this respect, but also confers upon it strong curare-like properties, as Crum Brown and Fraser

discovered for atropine itself (12). The other structural features which lead to atropine-like properties in alkamine esters are more restricted than those which confer curare-like properties and have already been discussed.

Local anaesthetic properties appear in alkamine esters when the acyl radical contains an aromatic nucleus and the basic group is tertiary. The anaesthetic activity, as Trevan and Boock showed (23), is a property of the free base and not of the cation of its salts; it is increased by conditions which favour hydrolysis of the salts, e.g. a higher pH of the solution or the use of a weak acid for salt formation, and is abolished by conversion of the anaesthetic drug into a quaternary salt. These conditions are probably necessary in order to allow the drug to penetrate the nerve sheath, but of the intimate process which blocks the passage of the nerve impulse nothing is known.

For clinical purposes a mydriatic should be readily absorbed from the conjunctival sac and for this reason a tertiary base will usually be preferred to a quaternary. The prolonged action of atropine is also probably due to its tertiary basic character because atropine metho nitrate does not exhibit it; a tertiary base may be firmly held by surface forces in the tissues whereas quaternary cations are not so readily adsorbed. At the same time promising clinical results have been obtained with one of our synthetic compounds, viz. E3, by Dr. Ida Mann and other workers; their results will be reported later.

SUMMARY

(1) Twenty-one synthetic compounds, all of them benzilic esters of alkamines, have been tested for mydriatic activity in the mouse; seven of them proved to be powerful mydriatics, comparable in intensity of action with atropine but shorter in duration.

(2) Fifteen of them were tested in the cat for atropine-like action in the salivary gland and on the blood pressure. The powerful mydriatics were all more active than atropine in both respects, and several compounds weaker than atropine in mydriatic activity were more active than atropine in the salivary gland.

(3) Several of the compounds were found to be atropine-like in smooth muscle and in the isolated cat's heart.

(4) Eight of the more active mydriatics were tested for toxicity; in mice all of them were more toxic than atropine on intraperitoneal or subcutaneous injection but two of the most promising compounds (E3 and P10) were less toxic than atropine on oral administration; the same two compounds produced much less severe symptoms on subcutaneous injection into cats than atropine did in equal doses.

(5) Benzilic esters of quaternary alkamines were in general more active in all the tests used than those of corresponding tertiary alkamines.

(6) The mydriatic activity of the quaternary metho-salts of atropine, l-hyoscyamine, l-hyoscine and eucatropine has been compared with that of their parent bases in the mouse pupil and after local application to the eye of the cat.

In the mouse the metho-salts, except l-hyoscine methiodide, are more active than the tertiary bases; in the cat the tertiary bases are the more active mydriatics.

(7) The relations between chemical structure and atropine-like action among the synthetic compounds and the belladonna alkaloids are discussed.

(8) Among the synthetic benzilic esters benzilyloxyethyl dimethylethyl ammonium chloride (E3) appears to be the most promising substitute for atropine in ophthalmic practice; its action equals that of atropine in intensity but is shorter in duration and its toxicity compares favorably with that of atropine.

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CONTINUOUS INTRAVENOUS CHEMOTHERAPY OF PLASMODIUM LOPHURAE INFECTION IN DUCKS[1]

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The assessment of antimalarial activity of drugs administered to ducks by the oral route is sometimes complicated by incomplete absorption from the intestine, or varying degrees of destruction of the drugs in the alimentary tract. In the method described below, white Pekin ducks are infected with *P. lophurae* and treated for three days with a continuous intravenous infusion containing the therapeutic agent. The precision of dosage afforded by this procedure has made possible the investigation of certain drugs and problems which are difficult to approach by the customary methods. The general usefulness of the method is illustrated by the following examples. Results of single daily intravenous doses of quinine and of cinchonine were compared with the results of continuous therapy with these drugs. Several compounds containing antimony or arsenic were examined, as well as two antibiotic agents. Finally, a comparison was made of the activities of some of the common antimalarial drugs and of their combinations.

APPARATUS AND METHODS. *Selection of ducks.* About 225–275 cc. of intravenous infusion fluid per kgm. per day was required to limit loss of body weight to less than 15% during the 3-day experiment; at rates above 300 cc. per kgm. per day, the ducks became somewhat edematous. It was necessary to inject fluid at a rate of at least 100 cc. per day to minimize clotting; at the arbitrarily chosen rate of 140 cc. per day, it was necessary to use birds of at least 500 grams in weight. Birds above 625 grams in weight were difficult to immobilize. Six ducks were selected within this weight range for an experiment. The recorded weight of each bird was 5% less than the observed weight, to allow for loss of weight through passage of fecal material during the first few hours of the experiment. The volume of liquid excreta collected during three days amounted to about 40–50% of the volume of the infusion injected.

Immobilization of birds. After wrapping the thighs of the ducks loosely with gauze to prevent trauma, the birds were arranged in a row on their backs on a tray 44 inches long by 20 inches wide which was mounted in a cabinet held at 30°C. The thighs rested in U-shaped metal stirrups; pins were passed through holes in the arms of the stirrups and over the legs of the birds to immobilize the upper portion of the legs. The webs of the feet were clamped to the front edge of the tray with screw-clamps. Holes were cut in the tray below the tails of the birds; vertical metal shields were slipped through these openings to deflect excreta to collecting pans arranged below the tray. It was desirable to slip a piece of sheet metal under the backs of the ducks; by elevating the sheet at the rear, the bodies of the birds were slightly flexed at the thighs, enabling a more nearly quantitative collection of excreta. When immobilized in this position, a duck does not retain food or fluid well when these are

introduced into the crop; consequently, water balance and nutrition must be maintained by the intravenous route.

Injection of infusion fluid. The needle for introducing the infusion into the leg vein of each duck was a 22 gauge hypodermic needle 0.5 inch in length, with the regular hub replaced by a smaller one. Coating of the inner surface of the needle with paraffine helped to reduce the incidence of clotting. The needles were introduced cleanly into the leg veins of the ducks and secured by adhesive straps; they were connected by light gum tubing to glass capillary tubes running to the individual injection pumps.

Six pumps were arranged on a common shaft, and each supplied an individual duck. The construction of one of the pumps is detailed in figure 1.
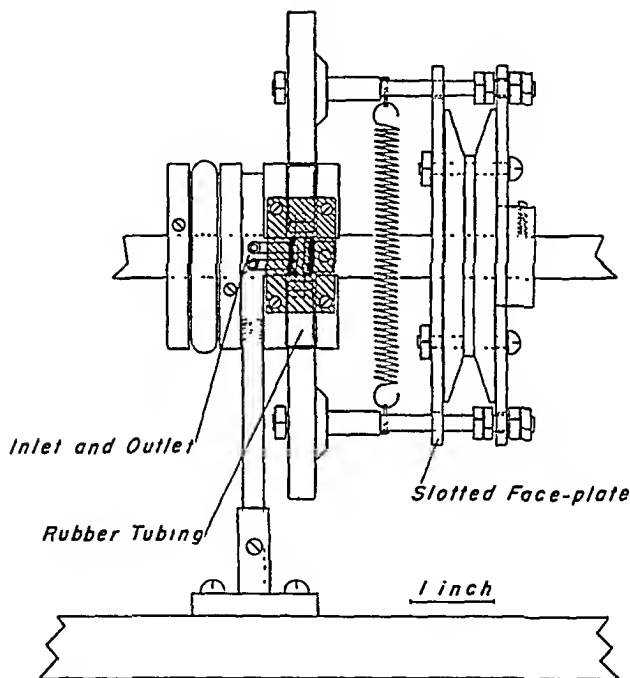
FIG. 1. INDIVIDUAL INJECTION PUMP

The pump consists essentially of a piece of rubber tubing, 0.125 inch in internal diameter and with walls 0 0625 inch thick, wrapped once around a fixed cylinder, with the ends of the tubing attached to a suitable metal inlet and outlet Two rollers, suspended diametrically opposite one another on a rotating element, were held firmly against the rubber tubing by two coiled springs, and occluded the tubing at the points of contact. As the rotating element was turned by the shaft, the rollers expressed the contents of the tubing through the outlet, and the tubing refilled behind the rollers from the inlet. The fixed cylinders were made from flat-face pulleys, each being fitted with two pairs of brass collars to form channels in which the rubber tubing lay. Each pulley carried the tubing for two pumps; a mounting pedestal prevented rotation of the pulley as the shaft revolved within it. The inlet and outlet tubes were 0.125 inch copper tubing, soldered into a piece of suitably drilled brass rodding fitted with nipples for attaching the rubber tubing   This

assembly was held in place by a mounting lug and screw, and was inset into the pulley so that it and the ends of the rubber tubing were covered by a curved brass plate attached to the brass collars. This cover-plate provided a smooth path to be traversed by the rollers as they passed over the gap between the ends of the tubing. If the plate is adjusted to lift slowly the advancing roller from the tubing, an uninterrupted outflow of liquid is obtained. A second pulley, attached to the drive-shaft by a setscrew, was equipped with face-plates as shown to hold the shafts carrying the rollers. The proximal plate was slotted, allowing radial movement of the roller-shaft, but not lateral motion of it.

The drive-shaft was driven at a speed of 7 revolutions per hour. The rubber tubing should be mounted on the pumps under moderate tension, and should be replaced after a month of continuous operation. Each pump delivered approximately 140 cc. per day, depending on the tension of the tubing. The daily rate of pumping of an individual pump did not vary from day to day from the mean value by more than 5%. The simplicity of construction and dependability of operation of this pump should make it useful in other types of experimental work.

Drug infusions. The reservoirs for holding drug infusions were graduated 500 cc. bottles fitted with Mariotte stoppers. The infusion was an isotonic solution containing 1.2% sodium citrate dihydrate and 4.2% glucose, adjusted to pH 7 by the addition of hydrochloric acid. In early experiments, a solution containing 0.8% sodium chloride and 0.8% sodium citrate was employed, but occasional ducks died on the second or third day. The infusion was prepared in a manner to insure sterility, and the pumps and tubing leading to the ducks were flushed out with alcohol.

One duck in an experiment received plain infusion; the others received drug infusions, the concentration of drug being determined by the desired dose, the weight of the bird, and the rated output of the individual pump. Injection of the infusions was begun at the time of infection.

Infection and criterion of response to therapy. Each bird was infected with an intravenous dose of 3×10^8 parasitized red cells per kgm. of body weight, the blood being taken from a large donor duck heavily parasitized with *P. lophurae.* A blood smear was prepared daily from each duck for estimation of the degree of parasitemia, and the volumes of infusions injected daily were recorded. The experiment was concluded on the third day, when the ducks were weighed again. The drug doses were recalculated on the basis of the mean weights during the experiment; a change of more than 10% from the intended dose was seldom encountered. The control birds reached a parasitemia of 40-70% of the red cells parasitized on the third day; rare experiments where the control did not reach 40% were discarded. The minimum effective dose of the drug was taken as the dose necessary to reduce the parasitemia in the treated bird to one-half that of the control, based on the third-day smears.

RESULTS. *Standardization with quinine.* Table 1 illustrates typical results obtained with various doses of quinine hydrochloride dihydrate; the minimum effective dose was read off a curve constructed from the data. In a series of experiments, the mean value of the minimum effective dose of the drug was 15 mgms. per kgm. per day, ranging from 13 to 17 mgms. per kgm. per day. The minimum effective doses of other drugs were determined in a similar manner; quinine hydrochloride in a dose slightly below and one slightly above the minimum effective dose was always included as a control.

Discontinuous therapy with quinine and cinchonine compared with continuous therapy. In table 2 are compared the effects produced by single daily doses and continuously administered doses of quinine hydrochloride by the intravenous route. The first single daily dose of drug was given at the time of infection. It is apparent that, at the level of minimum response, quinine was about twice as

effective on the basis of dosage when given once a day as when given by continuous infusion. On the other hand, the high degree of response produced by

TABLE 1

Response produced by quinine hydrochloride dihydrate against P. lophurae in the duck

Drug administered during a 3-day test in a continuous intravenous infusion of glucose-citrate.

DUCK NO.	DOSE	PER CENT ERYTHROCYTES PARASITIZED		
		1st day	2nd day	3rd day
	mgms. per kgm. per day			
1	0	9	11	61
2	0	10	17	65
3	11	7	11	39
4	14	10	12	34
5	18	4	8	3
6	20	5	4	2

TABLE 2

Responses produced by quinine hydrochloride dihydrate, administered intravenously once daily or continuously during a 3-day test, against P. lophurae in the duck

EXPERIMENT NO.	DOSE	FREQUENCY OF DOSAGE	PER CENT ERYTHROCYTES PARASITIZED		
			1st day	2nd day	3rd day
	mgms. per kgm. per day				
1	0		7	15	40
	11	once daily	5	9	21
	31	once daily	4	7	7
	21	continuoua	6	9	28
	30	continuoua	3	3	2
2	0		13	36	58
	5	once daily	9	27	59
	10	once daily	9	7	16
	15	continuous	14	13	27
	22	continuous	7	9	11
3	0		9	11	48
	21	once daily	3	2	0.5
	42	once daily	3	1	0.3
	42	continuous	3	0.7	<0.01
	82	continuous	3	0.06	<0.01

42 mgms. per kgm. per day of quinine given continuously was not equaled by the same dose given once daily.

At the level of minimum response, cinchonine was about equally effective when given once daily or by continuous infusion, the minimum effective dose

being in either case about 30 mgms. per kgm. per day. Death occurred in a large proportion of ducks injected with a single intravenous dose of 60 mgms. per kgm. of cinchonine, which prevented a comparison of the effectiveness of single and continuous doses of this drug at a high degree of response.

Compounds of arsenic and antimony. Table 3 expresses the results obtained with a few compounds of arsenic and antimony. The phenylarsinoxides were

TABLE 3

Activity of compounds of arsenic and antimony against P. lophurae in the duck

Drugs administered during a 3-day test in a continuous intravenous infusion of saline-citrate.

COMPOUND	MINIMUM EFFECTIVE DOSE
	mgms. per kgm. per day
Potassium antimony tartrate (tartar emetic)*	16
Sodium antimony thioglycollate†	16
3-NH₂-4-OC₂H₄OH-phenylarsinoxide*	inactive at 64
4-CH₃NHCOCH₃-phenylarsinoxide*	inactive at 16
4-OCH₂CONH₂-phenylarsinoxide*	inactive at 16
4-(CH₃)₂COOH-phenylarsinoxide*	inactive at 32
3-NH₂-4-OH-phenylarsinoxide (mapharsen)*	32
Quinine hydrochloride dihydrate	15

* Toxic at the indicated dose.
† Toxic at 32 mgms. per kgm. per day.

TABLE 4

Activity of antimalarials against P. lophurae in the duck

Drugs administered during a 3-day test in a continuous intravenous infusion of glucose-citrate.

COMPOUND	MINIMUM EFFECTIVE DOSE
	mgms. per kgm. per day
Quinine hydrochloride dihydrate	15
Cinchonine hydrochloride hydrate	30
Quinacrine dihydrochloride	6
6-Chloro-9-(2-diethylaminoethylamino)-2-methoxyacridine dihydrochloride	18
Pamaquine hydroiodide monohydrate	0.15

kindly supplied by Dr. Harry Eagle. The dosages of these drugs were in multiples of 10 mgms. per kgm. per day. Potassium antimony tartrate, sodium antimony thioglycollate, and mapharsen were active in doses at or near the toxic level. Tartar emetic is reported to be inactive in human malaria (1), and mapharsen is said to terminate clinical symptoms of malaria, but the blood smear remains positive (2).

Penicillin and streptomycin. Penicillin sodium (Pfizer clinical grade) was inactive at a continuous intravenous dosage of 59,000 units per kgm. per day. Several batches of crude penicillin were likewise found inactive in daily dosage of from 24,000 to 87,000 units per kgm. Penicillin is stated to be ineffective against human *P. vivax* infections (3).

TABLE 5

Summation of responses in simultaneous administration of quinine hydrochloride dihydrate and cinchonine hydrochloride hydrate

Drugs administered in a continuous intravenous infusion of glucose-citrate to ducks infected with *P. lophurae* during a 3-day test.

DUCK NO.	DOSE		PER CENT ERYTHROCYTES PARASITIZED		
	Quinine hydrochloride	Cinchonine hydrochloride	1st day	2nd day	3rd day
	mgms. per kgm per day	*mgms. per kgm. per day*			
1	0	0	9	17	85
2	15	0	11	12	14
3	0	35	6	10	4
4	7.5	17.5	8	16	14
5	10	23	4	4	4
6	15	36	2	1	0.4

TABLE 6

Failure of responses to summate in simultaneous administration of quinacrine dihydrochloride and pamaquine hydroiodide hydrate

Drugs administered in a continuous intravenous infusion of glucose-citrate to ducks infected with *P. lophurae* during a 3-day test.

DUCK NO.	DOSE		PER CENT ERYTHROCYTES PARASITIZED		
	Quinacrine dihydrochloride	Pamaquine hydroiodide	1st day	2nd day	3rd day
	mgms. per kgm. per day	*mgms. per kgm. per day*			
1	0	0	9	19	44
2	6	0	8	14	12
3	0	0.14	13	14	21
4	3	0 07	9	22	3S
5	4.3	0 10	10	15	39
6	6.5	0.15	8	15	11

Streptomycin hydrochloride (Merck) was inactive at a continuous dosage of *330,000* units per kgm. per day.

Quitenine. This drug is of interest because it is affirmed by some, and denied by others, to be a degradation product of quinine in man (4). It was found to be inactive in a continuous dose of 72 mgms. per kgm. per day, which agrees with the observation that it is inactive in human malaria in a dose higher than the effective dose of quinine (4).

Activity of some common antimalarial drugs and their combinations. The minimum effective doses of these drugs were determined as described previously for quinine; the results are given in table 4. The responses produced by combinations of some of these drugs in pairs were investigated. The dosages of the six ducks in such a combined therapy experiment were as follows: (1) untreated control; (2) minimum effective dose, (a), of drug A; (3) minimum effective dose, (b), of drug B; (4) 0.5 (a) plus 0.5 (b); (5) 0.67 (a) plus 0.67 (b); (6) 1.0 (a) plus 1.0 (b). On the basis of the results, the pairs of drugs could be classified as exhibiting either summation of response or no summation of response. Summation of response was observed when the response in bird no. (4) was equal to, and that in bird no. (6) was considerably greater than, that in birds no. (2) or no. (3). In table 5 is presented the protocol of a typical case where summation was observed. When no summation was observed, as in table 6, the responses were of the magnitude produced if only one drug of the pair were present. Summation was observed for quinine-cinchonine, and for quinacrine-6-chloro-9-(2-diethylaminoethylamino)-2-methoxyacridine, which is to be expected on the assumption that, in the respective pairs, both drugs have a common mechanism of action. No summation was seen with quinine-quinacrine, quinine-pamaquine, or quinacrine-pamaquine. Assuming that one drug of a pair has no effect on the disposition of the other drug at the dosage level employed, these results suggest that three different principal mechanisms of drug action against *P. lophurae* are involved for quinine, quinacrine, and pamaquine. This does not exclude the possibility that these drugs may have common secondary mechanisms of action.

SUMMARY

1. A method incorporating a simple rotary injection pump is described for continuous intravenous administration of drugs to ducks infected with *P. lophurae.*

2. On the basis of dosage, quinine was more effective at the level of minimum therapeutic response when given intravenously in single daily doses than when given by continuous infusion; at a high level of response, the converse was true

3. At the level of minimum response, cinchonine was equally effective when given in single daily intravenous doses or continuously.

4. Tartar emetic, sodium antimony thioglycollate, and mapharsen were effective in doses at or near the toxic level.

5. Penicillin, streptomycin, and quitenine were inactive in the doses employed.

6. Summation of therapeutic response was shown by the following two combinations: quinine-cinchonine, and quinacrine-6-chloro-9-(2-diethylaminoethylamino)-2-methoxyacridine. No summation of response was exhibited by the following combinations: quinine-quinacrine, quinine-pamaquine, and quinacrine-pamaquine. These results suggest that quinine, quinacrine, and pamaquine each possess a different principal mechanism of drug action against *P. lophurae* in the duck.

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A SHORT-TERM CHRONIC TOXICITY TEST EMPLOYING MICE[1]

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When large numbers of synthetic organic compounds were examined in this laboratory for antimalarial properties, the determination of toxicity of the more active drugs was an aid in orienting the program. The amount of compound available for toxicity investigation was seldom more than five to seven grams. A chronic toxicity determination was indicated, since successful compounds would be employed in man in multiple daily doses for a number of days.

Oral administration of drugs by the drug-diet method was employed, as it appears to be the most convenient method for frequent dosage of mice (1). There are numerous reports in the literature in which the rat has been used for chronic toxicity investigations, the drugs being administered by drug-diet, and the inhibition of weight-gain being used as an index of toxicity. The mouse was chosen as an experimental animal for the present work in preference to the rat for the following reasons: (1) a simple means is available for administering drug-diets quantitatively to the mouse; (2) the smaller size of the mouse permits the use of more animals for a given quantity of drug; and (3) occasionally, drugs appear to be so obnoxious to the rat that satisfactory drug intakes are not attained (2).

The method described below determines the maximum tolerated daily dosage of the drug administered for one week to groups of ten mice. The relatively short treatment period of seven days was a limitation imposed by the moderately low toxicity of the drugs examined and the limited quantity available. Nearly two hundred compounds have been examined by this method; based on those which have reached the stage of limited clinical trial in man, the following generalizations can be made concerning the relationship between the toxicity of these compounds in the mouse and the human: (1) The mouse test frequently enables a rough "order of magnitude" of toxicity of a new compound to be assigned. Pamaquine (plasmochin), for instance, is seen to be more toxic than quinacrine (atabrine), which in turn is more toxic than quinine, and this is likewise true in the human. Due to well-recognized differences between the two species, fairly large discrepancies can be expected to occur. (2) In several series of chemically closely related compounds (e.g., homologous series), the relative toxicities of the members of each series have thus far agreed quite well in the mouse and the human.

Experimental method. Special cages were designed to minimize attention during the course of an experiment. A cage 6 inches high by 6 inches deep by 25 inches long was folded from 24 gauge galvanized iron. It was fitted with a bottom grid of wire mesh (16 mesh to the square inch), and a set of legs raising the grid 1.25 inches off the supporting surface. The cage cover was hinged in the center in the long dimension, the rear segment being made of sheet metal, and the front segment of wire mesh. The cage was divided by nine partitions into ten individual cages measuring 6 inches high by 6 inches deep by 2.5 inches wide. Through a rectangular hole in the narrow front side of each individual cage was inserted the horizontal leg of a food hopper constructed as shown in figure 1 from 28 gauge galvanized iron. The bottom of the food hopper rested on the wire grid floor, and the hopper was permanently attached to the cage wall. The edge of the half-inch hole through which the mouse consumed food was rolled to prevent injury. A strip of metal, soldered around the edge of the platform pierced by the feeding hole, served as a retaining wall to help prevent wastage of food. Finally, a narrow trough, 1.4 inches wide by 0.75 inches deep by 24.5

1 inch
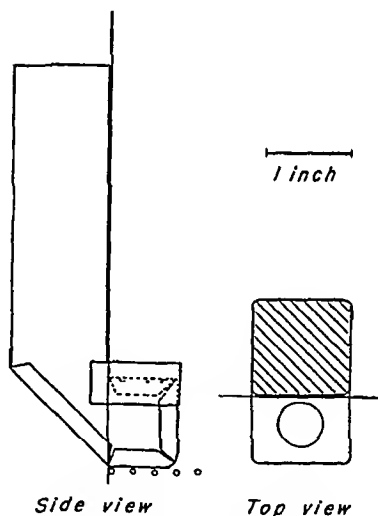
Side view   *Top view*
FIG. 1. MOUSE FOOD HOPPER

inches long, was suspended just under the grid of the cage below the row of food hoppers to catch any food spilled off the feeding platform. The maximum food wastage in this cage was 8%; usually, it was about 4%. The solid section of the hinged cage cover was drilled at proper intervals to admit the stems of conventional water feeders constructed from 100 cc. wide-mouth bottles. The food hopper and water bottle as described hold a 10–14 day ration for a 20 gram mouse.

Drug-diets were prepared in 300 or 400 gram quantities for each group of 10 mice, depending on the anticipated toxicity of the particular diet. The drug sample was ground thoroughly with 5–10 grams of starch as an excipient, and powdered Wayne Fox chow was slowly ground in to give the desired final diet concentration, followed by a thorough mechanical mixing. If a very limited amount of drug were available, a single diet was prepared of sufficient concentration to be intolerable to the mice; successive dilutions were made from the residual diet until a tolerated diet was found. Each successive diet concentration was not less than one-half the preceding concentration.

Each diet concentration in an experiment was offered to a group of 10 mice contained in one of the special cage units. The total available diet was weighed, and a small weighed amount of diet was reserved to replenish food hoppers exhausted before the end of the experiment; the rest of the diet was distributed equally by weight into the ten food hoppers. Ordinary household measuring cups, if filled and leveled by a standard procedure, served admirably to divide the food equally, as the error in weight delivered to each hopper was less than five per cent of the mean weight delivered to each of the ten hoppers.

Mice weighing 16 ±2 grams were most satisfactory; animals weighing less than 14 grams did not grow well in the cages, and mice above 18 grams in weight frequently restricted their consumption of drug-diets.

The mice used were the CFW strain (Carworth Farms), and were generally males. They were held in the laboratory several days, and on the day of the start of the experiment, they were divided into three groups weighing 14–16, 16–18, and 18–20 grams. Any animals appearing less lively than normal, or showing signs of diarrhea, were discarded. The desired number of samples of 10 mice were made up by aliquots from these large groups, in order that roughly equal weights for all samples be obtained. There should not be more than one or two mice in the 10-mouse sample weighing 18–20 grams. If each mouse is weighed when the animals are transferred to the cages, individual mouse responses can be calculated and some idea gained of the variability within the sample. In spite of this advantage, for practical purposes it suffices to merely weigh the total mouse sample, and the procedure to be described is based on this method of treatment. The mice must not be excessively hungry when placed in the cages.

The cages were held during the experiment in a closed room with lights controlled by a timer in a sequence of 3 hours light, followed by 3 hours of darkness. Only a slight smoothing of feeding habits is gained by this imposed 6-hour day. The room was not air-conditioned, nor was the temperature held closer than about ±4°C. during an experiment. Although environmental temperature conditions the acute toxicity of drugs in mice (3), there has been no consistent variation in toxicity of drugs in this test over the winter-summer temperature range encountered (10°–30°C.).

The day of death and the weight of each mouse dying during the experiment were recorded, as well as the presence of any obvious cause of death. Each day, enough drug-diet was pushed down from the vertical portion of the food hopper into the horizontal portion to replace that consumed by the mouse. In order to minimize wastage, the level of the diet was kept at least one-eighth of an inch below the edges of the feeding hole.

On the seventh day, the experiment was terminated. The survivors in each mouse group were combined, and the mean weight of the survivors was determined. Ten shallow U-shaped troughs, each 2.5 inches wide, were laid side to side, and the narrow trough under the food hoppers was emptied over the set, so that the diet spilled by each mouse was collected separately. Any fecal contamination was removed by sifting. Then the diet remaining in the food hoppers was collected in similar fashion. The unused diet portion of each mouse dying during the experiment was weighed separately, and the unused diet of the survivors of each group was combined and weighed.

INTERPRETATION OF RESULTS. In this test, it was considered desirable to select an objective criterion of toxicity which was sufficiently well-defined as to permit the assignment of a numerical toxicity value to each compound encountered. Not all criteria which have been employed in evaluating toxicity in larger animals apply to the present circumstances. The drug-induced changes in behavior and appearance of the mice in this work have generally been difficult to evaluate objectively, due in large part to the small size of the animal. Rare compounds produced characteristic changes, such as ataxia, paralysis, diarrhea, or edema. With the usual compound, the mice either appeared normally active up to the point of death if they experienced little weight-loss, or lethargic if

there had been considerable loss of weight.   Compounds which in larger animals produce acute symptoms of central nervous system stimulation or depression did not invariably show characteristic symptoms in this test.

Investigation of gross and microscopic changes in tissues was not done, due to lack of facilities for handling the large amount of material.   It is questionable whether the short treatment period would have permitted full development of characteristic pathological lesions for most compounds.

The inhibition of growth of a mouse following administration of drugs appears to be much more erratic than that of a rat, and we have used the change in weight of the 10-mouse group only as a supplementary piece of evidence in evaluation of toxicity.
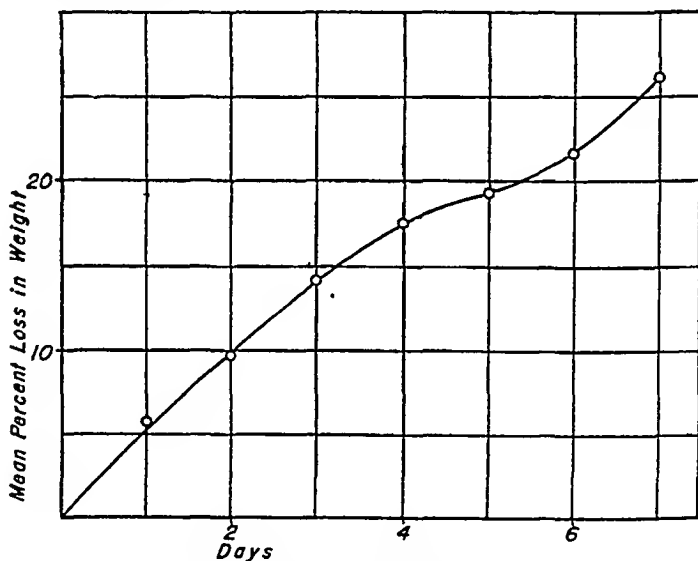
Fig. 2. Rate of Weight Loss in a Group of 10 Mice at the Maximum Tolerated Drug Intake Level of Quinacrine

Death has proved to be the most reliable index in the present test.   The objective is to select the maximum mean daily drug intake which allows the survival for 7 days of at least nine out of ten mice.   It is necessary to decide the cause of death of each mouse.   Using the final combined weight of surviving mice in a group, the number of mouse days, and the total diet consumption for survivors, the mean daily diet intake per kilogram of body weight per day is calculated.   Likewise, this value is calculated for each mouse dying during the experiment.   A mouse which has eaten far less than the survivors can be assumed to have died of inanition; usually this is due to dislike of the diet, and such deaths occur from the second to the fifth day.   A mouse which is excessively hungry when offered the drug-diet may consume enough to produce acute

toxicity and death. Such a mouse will usually have a larger diet intake than the mean for the group, and death will occur early in the experiment. Mice dying from either of these two causes are discarded from the experiment. Mice are retained which have died with diet consumptions not far removed from the mean value. Figure 2 illustrates the uniform rate of weight loss for a 10-mouse group typically seen at the maximum tolerated dosage level of a compound producing severe weight loss, such as quinacrine hydrochloride, and indicates that the mean weight of the group during the experiment is to be preferred for calculation of the mean diet intake. Using this mean weight value, a new mean value for diet
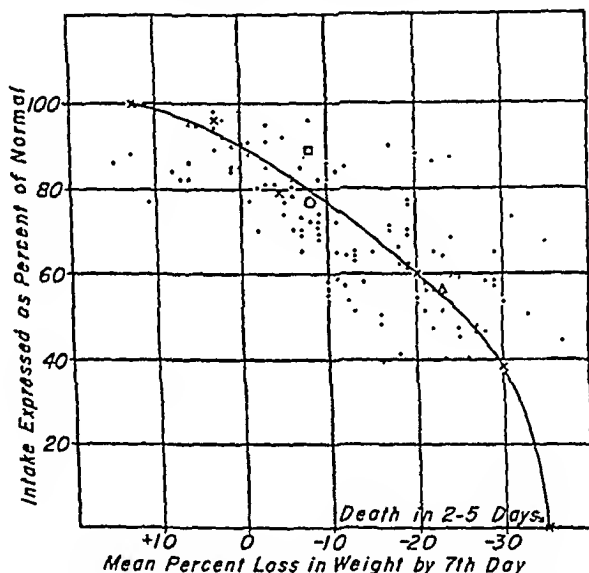
FIG. 3. WEIGHT LOSS OF MICE ON UNRESTRICTED AND RESTRICTED NORMAL DIET AND ON MAXIMUM TOLERATED DRUG-DIETS

—X— Mice offered various amounts of normal diet. Drug diets: O quinine; △ quinacrine; □ pamaquine; ● results with 113 other drugs.

consumption is calculated for significant mice in the group, and from the diet percentage, one arrives at the mean drug intake.

A perplexing question arose in connection with compounds which cause a marked loss of weight: how much do inanition and drug toxicity separately contribute toward death? Six groups of six mice were offered normal diet in amounts ranging from none to unrestricted quantities. The mean percentage weight change of each group is related in figure 3 to the diet intake expressed as a per cent of the normal unrestricted intake. If the intake was less than 30% of normal, death occurred in a high percentage of cases within seven days. On the same figure have been placed the values at the maximum tolerated drug intake level for 116 compounds; the normal diet intakes on which some of the values

are based were estimated from non-toxic drug diets in the experiments. In about ten per cent of the compounds examined, the degree of inanition may well have been the major factor in the death of the animals. Figure 3 also indicates that the use of the criterion of weight loss would have led in a large majority of cases to a toxicity value different from that based on death.

A criticism that has been made against the administration of drugs in the diet in toxicity work is that the animals may exhibit more inanition than if the drugs were given by stomach-tube. Quinacrine dihydrochloride is a compound which produces severe weight loss and inanition when administered in the diet (fig. 3), and if the criticism is justified, a difference in food consumption might be ex-

TABLE 1

Comparison of seven-day chronic toxicity of quinocrine hydrochloride when administered by drug-diet ond by stomach-tube

DRUG ADMINISTRATION	MEAN PER CENT LOSS IN WEIGHT	DEAD/TOTAL ON 7TH DAY	MEAN DIET INTAKE	MEAN DRUG INTAKE
			grams/kgm./day	grams/kgm /day
0.25% drug-diet................	21	1/10	142	0.36
0.125% drug-diet............	11	0/10	162	0.20
0.0625% drug-diet................ ..	4	0/10	202	0.13
Tubed once daily...........		10/10	54	0.60
Tubed once daily.	22	2/10	129	0.34
Tubed once daily..................	11	0/9	182	0.16

TABLE 2

Voriotion in meon drug intakes of individual mice at maximum toleroted drug-diet concentrations odministered for 7 doys

DRUG	NUMBER OF MICE	DRUG INTAKE		STANDARD DEVIATION
		Range	Mean	
		grams/kgm./day	grams/ kgm./day	
Quinine hydrochloride dihydrate	18	1.15–2.3	1.64	0.23
Quinacrine dihydrochloride....	20	0.24–0.64	0.41	0.10
Pamaquine hydroiodide monohydrate	18	0.09–0.135	0.119	0.014

pected if the drug were given by stomach-tube. Table 1 indicates that no significant differences in mortality, weight loss, or food consumption were seen when the two methods of administration were employed. Furthermore, the mice which died on both regimes ate practically no food for a day preceding death.

PRECISION OF TEST. Table 2 gives an idea of the typical variation in drug intake of individuals in mouse groups offered the maximum tolerated drug-diet concentration of quinine hydrochloride dihydrate, quinacrine dihydrochloride, and pamaquine hydroiodide monohydrate for a 7-day period. In a group of 20

mice fed normal diet, the grams of diet consumed per kilogram per day ranged from 161 to 240, the mean was 211, and the standard deviation was 22.

Table 3 illustrates the variability in successive determinations of the maximum tolerated drug intake for these three drugs. In these experiments, the drug concentration in the maximum tolerated diet was usually 50% higher than in the next lower diet; the 50% change in concentration was usually accompanied by only a 20-30% change in drug intake, due to increased inanition of the mice at the higher diet concentration. It appears unlikely that the use of smaller drug-diet concentration increments would be accompanied by any substantial gain in precision.

As in other bioassay methods, it was observed that when two compounds were compared in successive tests, the ratio of toxicities of the pair was more constant than the absolute toxicities. For example, in four comparisons of quinine and quinacrine, the maximum tolerated drug intakes (grams per kgm. per day) and their ratio were as follows: 1.43, 0.395, 3.6; 1.88, 0.45, 4.2; 1.34, 0.35, 3.8; and 1.64, 0.40, 4.2. The highest value of tolerated drug intake was 40% and 29%,

TABLE 3
Variability in determinations of maximum tolerated drug intakes in successive 7-day experiments

DRUG	NUMBER OF DETER-MINATIONS	MEAN % LOSS IN WEIGHT	TOLERATED DRUG INTAKE		STANDARD DEVIATION
			Range	Mean	
			grams/kgm /day	*grams/ kgm /day*	
Quinine hydrochloride dihydrate..	36	9	1.23 –2.22	1.68	0.26
Quinacrine dihydrochloride.. .	7	20	0.300–0.451	0.368	0.049
Pamaquine hydroiodide mono-hydrate .	9	9	0.072–0.133	0.098	0.021

respectively, above the lowest value for each of the two compounds, whereas the highest ratio of toxicities obtained was only 17% above the lowest value. For this reason, we have employed a suitable compound in each experiment to serve as a standard of reference, against which the toxicities of the new compounds were expressed as ratios. The standard of reference was set up so that the maximum tolerated diet concentration was 50% greater than the next lower diet concentration. If the new compounds are set up in like manner, it is believed that differences in toxicity between two compounds of the order of twenty-five per cent may be detected, using ten mice for each diet concentration.

In this test, quinacrine hydrochloride was four times as toxic as quinine hydrochloride, and pamaquine hydroiodide was fourteen times as toxic as quinine hydrochloride.

SUMMARY

A method is described for determining the chronic toxicity of drugs when administered to mice for seven days. The use of the drug-diet method of ad-

ministration, and of death as an end-point, results in a simple procedure for rapid evaluation of large numbers of compounds. The precision of the method is illustrated by data on quinine, quinacrine, and pamaquine.

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THE CENTRAL STIMULANT ACTION OF SOME VASOPRESSOR AMINES

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Stimulation of the central nervous system is generally recognized as an important part of the action of certain vasopressor amines. While this action may be valuable for some clinical uses it may be more or less undesirable in others. The importance of central activity then, as well as the lack of information for several compounds recently introduced into medical practice, led to the present study.

The central stimulant activity of seven commercial vasopressor amines was estimated by determining the total activity of white rats. The technique employed was similar to that of Schulte and co-workers (1, 2) except that in the present study the doses employed were fractional parts of the LD_{50}. Such a dosage plan is reasonable since in evaluating drugs considerable importance is attached to the relation between various effects and toxicity as determined by lethal doses. In addition to providing data on the comparative central stimulant action, the present study yields information regarding satisfactory dosage levels for evaluating new compounds.

METHOD. Adult white male rats weighing 190 to 230 grams, the weight range used in the acute subcutaneous toxicity studies at 26°C., were employed in these experiments. Sixteen individuals were used for each dosage level. These animals were divided into two groups of eight rats each which were tested on successive days. Doses equal to five, ten, or twenty per cent of the LD_{50} were administered subcutaneously on the dorsal surface. Animals were then put into the test chamber at 29°C., and the activity was recorded for a period of six hours. The average activity of the sixteen animals for each test was determined, and the standard error was calculated.

Control rats were given physiological saline solution subcutaneously in a volume equivalent to that administered in the highest dosage level. No group of rats was used more than once in order to eliminate any possible effects from previous injections.

The amines studied in this investigation were: Benzedrine (*dl*-1-phenyl-2-aminopropane); Ephedrine (*l*-1-phenyl-2-methylaminopropanol-1); Propadrine (*dl*-1-phenyl-2-aminopropanol-1); Vonedrine (*dl*-1-methylamino-2-phenylpropane); Privine (2[α-naphthylmethyl] imidazoline); Tuamine (*dl*-2-aminoheptane); and Neo-Synephrine (1-[m-hydroxyphenyl]-2-methylaminoethanol).

RESULTS. It will be seen from table 1 that Privine, Tuamine, and Neo-Synephrine caused little if any significant increased activity in any dose level. Vonedrine had no significant effect at the lowest dose level and only slight effect at the highest level. Benzedrine, Ephedrine, and Propadrine were the active compounds. The relative activity of these three varied with different

dose levels. Benzedrine was most active at the high level, Ephedrine and Propadrine at the intermediate, and Ephedrine at the low dose level.

From these results it is apparent that the maximal stimulating effect of Ephedrine was obtained with five per cent of the LD_{50}, and maximal effects of Propadrine and Benzedrine were obtained with ten and twenty per cent respec-

TABLE 1

The average activity of rats following subcutaneous administration of vasopressor amines in doses equal to fractional parts of the LD_{50}

COMPOUND	LD_{50}*	REVOLUTIONS OF WORK ADDED AT EACH DOSE LEVEL		
		5 per cent	10 per cent	20 per cent
	mgm./kgm.			
Benzedrine..............	180	41.38 ± 4.34	52.75 ± 5.60	151.50 ± 19.16
Ephedrine	800	71.00 ± 10.26	74.95 ± 9.50	72.81 ± 8.50
Propadrine.	850	21.88 ± 3.68	75.25 ± 10.33	68.63 ± 8.30
Vonedrine	850	10.25 ± 1.76	17.80 ± 1.80	25.55 ± 3.38
Privine	420	9.69 ± 1.25	12.25 ± 1.87	16.75 ± 2.85
Tuamine	130	8.00 ± 0.92	6.69 ± 1.15	13.95 ± 3.01
Neo-Synephrine...	28	9.38 ± 1.76	4.90 ± 0.48	5.88 ± 0.94
Control........				9.25 ± 2.50

* These values were determined by inspection of mortality curves. Treatment of the data according to the method of Miller and Tainter (3) gives slightly different values, but the differences are not significant.

All compounds were tested as the hydrochloride salts except Tuamine which was tested as the sulfate.

TABLE 2

Mortality ratios for various vasopressor amines in rats injected subcutaneously with fractional parts of the LD_{50} (determined at 26°C.) and kept at 29°C.

COMPOUND	PART OF ORDINARY LD_{50}		
	5 per cent	10 per cent	20 per cent
Benzedrine................... ..	0/16	1/16	5/16
Ephedrine......	1/16	4/16	9/16
Propadrine......	1/16	3/16	13/16
Vonedrine....	0/16	0/16	0/16
Privine.........	0/16	0/16	0/16
Tuamine.......	0/16	0/16	1/16
Neo-Synephrine..........	0/16	0/16	0/16

tively of the LD_{50}. This relation between dosage and response is in agreement with that noted by Shulte and co-workers (1). They found that 20 mgm./kgm. of Benzedrine and 20 mgm./kgm. of Ephedrine were required to produce the maximal total effect while 80 mgm./kgm. of Propadrine was necessary to give this response.

The hourly activity records showed that generally the greatest activity occurred during the first or second hour of the test. There was a rapid decrease in activity after the peak was reached so that generally by the end of the test period no stimulation was recorded. Benzedrine and Ephedrine showed a more persistent effect. Rats which received the higher doses of these amines still exhibited some increased activity after six hours.

The results of these tests indicate that doses based on fractional parts of the LD_{50} are satisfactory for evaluating comparative central stimulant action. The most suitable dosage level for screening tests appears to be twenty per cent of the LD_{50}. Reactions to this amount of the amines, as well as extent of the information desired, will then determine whether the dosage levels should be increased or decreased.

Although only fractional parts of the LD_{50} as determined at 26°C. were administered, data presented in table 2 show that some of the amines became more toxic under the conditions of these experiments.

Studies, which will be reported later, indicate that the increase toxicity is related to the increased environmental temperature employed in the constant temperature chamber.

SUMMARY

The subcutaneous administration of seven commercial vasopressor amines in doses equal to fractional parts of the LD_{50} as determined at 26°C. showed that Benzedrine, Ephedrine, and Propadrine caused the greatest central stimulant action as measured by the total activity of white rats.

Maximal stimulating effects observed for these three compounds were obtained with five, ten, and twenty per cent of the LD_{50} of Ephedrine; ten and twenty per cent of the LD_{50} of Propadrine; and twenty per cent of the LD_{50} of Benzedrine.

Vonedrine caused a small amount of stimulation in the higher doses while Tuamine, Privine, and Neo-Synephrine had no significant effect in the doses employed.

Twenty per cent of the LD_{50} seems to be the most suitable dosage level for preliminary screening tests since this dose alone produced maximal observed effects with all compounds.

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OBSERVATIONS ON THE NATURE OF THE ANTAGONISM OF HISTAMINE BY β-DIMETHYLAMINOETHYL BENZHYDRYL ETHER (BENADRYL)[1]

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It has been shown that several of the benzhydryl ethers are effective in preventing fatal experimental asthma in guinea pigs subjected to a fine spray of histamine solution (1). These ethers have also been shown to decrease the severity of anaphylaxis in the guinea pig (2). Of the substances tested in this fashion, beta-dimethylaminoethyl benzhydryl ether (benadryl) was the most effective.
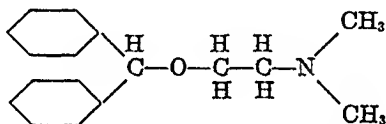
(Beta-dimethylaminoethyl Benzhydryl Ether)

Such a drug as this would be useful in evaluating the importance of histamine in various experimental and clinical situations in which it is known or suspected that histamine contributes to the pathogenesis (e.g., anaphylaxis, peptone shock, trypsin shock, urticaria, asthma, hay fever, etc.). However, before such an evaluation can adequately be performed it is necessary to know the magnitude and if possible the mechanism of this histamine antagonism. Such has been the purpose of the present investigation, a preliminary report of which has been made (3).

Under conditions of uniform anesthesia and constant blood pressure, histamine will, if injected intravenously in submaximal doses at suitable intervals, cause quantitatively reproducible falls in blood pressure in the dog. This response is sufficiently delicate and constant to have allowed it to be employed in the biological assay of histamine, and in the present instance makes it possible to study the effect of the proposed antagonist, benadryl, in one and the same animal, obviating the necessity for statistical comparison of treated and control animals.

Preliminary observations indicated that intravenous injection of benadryl (3 mg. per kilo as the hydrochloride) into barbital anesthetized dogs almost completely abolished the fall in blood pressure produced by 0.001 to 0.002 mg.

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per kilo of histamine acid phosphate. The reduction of the blood pressure response to larger doses of histamine (0.016 to 0.032 mg. per kilo) did not appear to be as marked. However, when an attempt was made to duplicate, after benadryl, the initial fall in blood pressure produced by 0.016 to 0.032 mg. per kilo of histamine, the dose of histamine had to be substantially increased, indicating that considerable inhibition had occurred.

A = histamine injected prior to benadryl
B = histamine injected following 3.0 mg. per kilo of benadryl
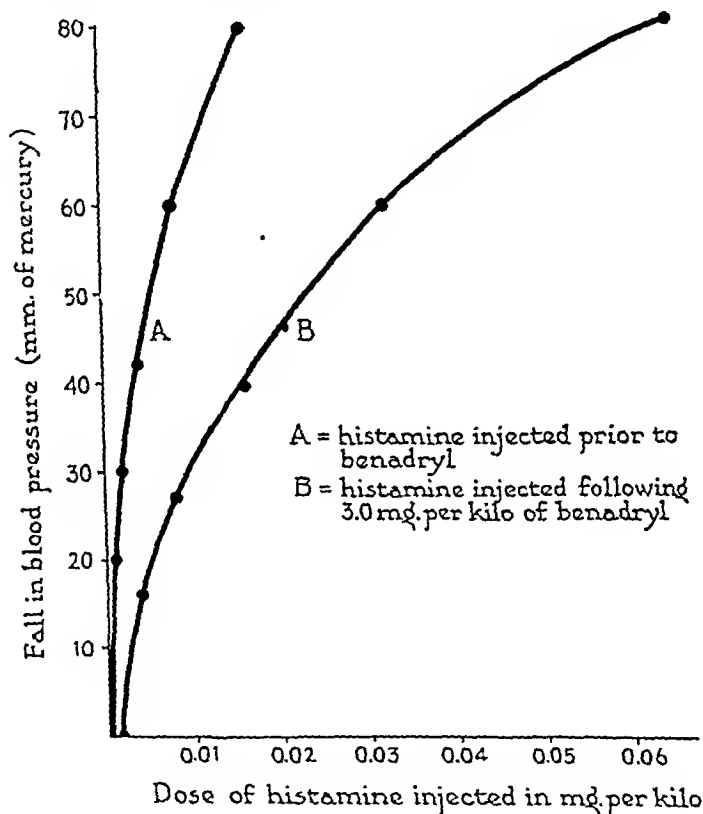
Fig. 1

Due to the fact that the dose-effect curve for histamine is not linear (fig. 1-a), it is obvious that the degree of change in the blood pressure response to histamine is not the optimal index of the antagonistic activity of benadryl. It, therefore, became necessary to determine how much of a given dose of histamine was antagonized by benadryl. This can be accomplished by attempting to reproduce with histamine, following the injection of benadryl, the fall in blood pressure produced by a dose of histamine given prior to benadryl. The difference between these two doses represents the amount of histamine antagonized.

A series of experiments was conducted in which histamine acid phosphate was injected in doses of 0.001, 0.002, 0.004, 0.008, 0.016 and 0.032 mg. per kilo. The maximum fall in blood pressure produced by each dose of histamine was measured in millimeters of mercury. Benadryl (3 mg. per kilo) was injected intravenously, and after five minutes progressively increasing doses of histamine were injected in an effort to reproduce the range of blood pressure depressions produced by the original doses of histamine (fig. 2).

In order to determine the amount of a given dose of histamine which was antagonized by benadryl curves were constructed relating the fall in blood pressure (in mm. of mercury) before (fig. 1-a) and after (fig. 1-b) the injection of benadryl. The amount of a given dose of histamine which is antagonized by benadryl can be determined from these curves by subtracting from the dose of histamine injected following benadryl, the dose of histamine which produced the same fall
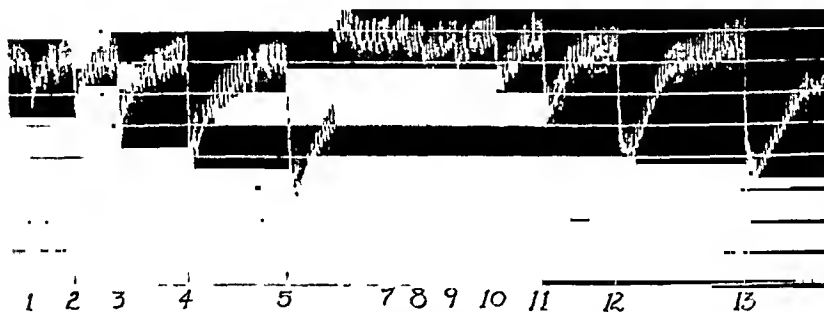
FIG. 2. DOG, 10 KILO; BARBITAL ANESTHESIA, CAROTID BLOOD PRESSURE

1—0 001 mgm. per kilo histamine acid phosphate intravenously, 2—0 002 mgm per kilo; 3—0 004 mgm. per kilo, 4—0 008 mgm per kilo, 5—0 016 mgm per kilo, between 5 and 7 3 0 mgm. per kilo of benadryl intravenously, 6—0 001 mgm per kilo of histamine acid phosphate per kilo intravenously, 8—0 002 mgm per kilo, 9—0 004 mgm per kilo, 10—0 008 mgm per kilo, 11—0 016 mgm per kilo, 12—0 032 mgm per kilo, 13—0 064 mgm per kilo.

in blood pressure prior to benadryl. Inspection of these curves reveals an apparent relationship between the doses of histamine which produce a given fall in blood pressure before and after benadryl. In each instance a constant multiple of the original dose of histamine seemed to be required. In order to test whether such a relationship actually exists, a plot was made of the dose of histamine injected following benadryl against the amount of this dose which was antagonized by benadryl. Such a plot (fig. 3) reveals a direct proportionality. Or, in other words, a constant per cent of any dose of histamine appears to be antagonized by a given dose of benadryl.

Such a relationship suggests that benadryl antagonizes histamine by interfering with an equilibrium between histamine and its site of action, by combining either with histamine or its site of action   The chemical structure of benadryl (beta-dimethylaminoethyl benzhydryl ether) suggests that direct chemical interaction with histamine is improbable, leaving combination of benadryl with the

site of action of histamine and subsequent interference in the normal histamine equilibrium a strong possibility.

In view of the direct proportionality between the dose of histamine injected and the amount of this dose antagonized by a given amount of benadryl, it be-
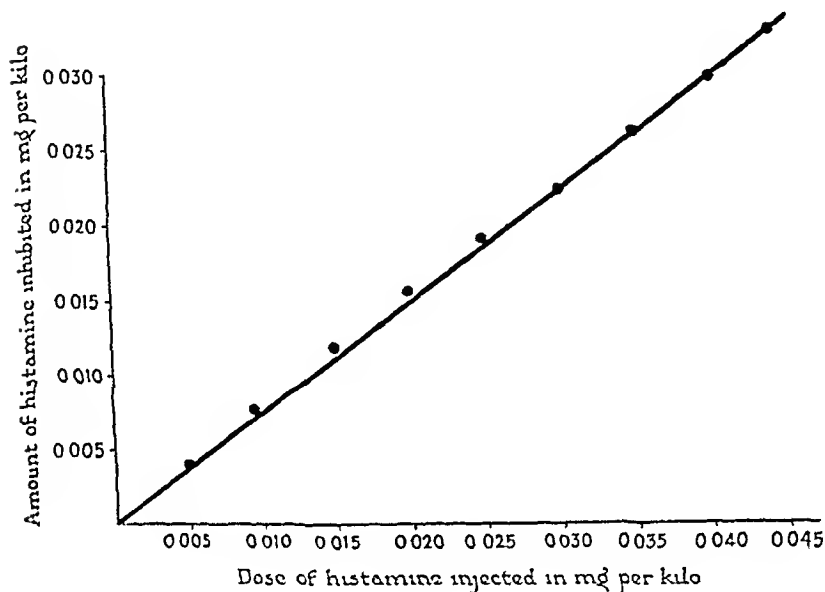
FIG. 3

FIG. 4

DOSE OF HISTAMINE	FALL IN BLOOD PRESSURE (MM OF MERCURY) AFTER INDICATED AMOUNTS OF BENADRYL (MG PER KILO)								
	0	0 1	0 2	0 5	1 0	2 0	4 0	8 0	16 0
mg per kilo									
0 001	34	20	16	12	6				
0 002	44	34	32	20	14				
0 004	60	42	38	34	22	20			
0 008	66	56	50	46	38	26	20		
0 016	72	64	60	58	50	38	28	26	
0 032					56	48	40	40	32
0 064					70	62	52	50	46
0 128							64	56	50

comes important to know how this constant changes with varying doses of benadryl. It was previously noted that the histamine antagonizing effect of a dose of 3 mg. per kilo of benadryl persisted with only slight diminution for a period of two hours after injection. It was, therefore, assumed that any addi-

tional injection of benadryl during such an interval would result in more or less complete cumulation, and thus it would be possible to study the influence of increasing concentrations of benadryl in the same animal.

In a series of experiments on four dogs progressively increasing doses of histamine acid phosphate were injected intravenously to determine the control blood pressure responses to histamine. Following the injection of 0.1 mg. per kilo of benadryl, varying doses of histamine were again injected and showed decreased effects. This procedure was repeated for additional doses of 0.1, 0.3, 0.5, 1.0, 2.0, 4.0 and 8.0 mg. per kilo of benadryl, making cumulated concentrations of 0.1, 0.2, 0 5, 1.0, 2.0, 4.0, 8.0 and 16.0 mg. per kilo of benadryl. The results of one such experiment is shown (fig. 4).

By the previously mentioned technique, the per cent antagonism of histamine was determined for three doses of histamine at each effective concentration of benadryl. The values for the per cent antagonism of the various doses of his-

Fig. 5

EFFECTIVE CONCENTRATION OF BENADRYL	PER CENT INHIBITION OF HISTAMINE				
	Exper. 1	Exper 2	Exper 3	Exper 4	Average
mg per kilo					
0.1	61 4	42 4	64 6	53.0	55.2
0 2	71 2	61 2	76 9	64 7	68.5
0 5	81 0	74 3	85 8	70 7	77.9
1 0	88 4	81 3	88 9	82 3	85.2
2 0	93 0	87 7	92 0	87 3	89 9
4 0	95 8	92 7	93 6	93 1	93 8
8 0	96 3	94 4	94 0	95 5	95.0
16 0	97 3	96 4		97 5	97 1

tamine varied only slightly and were averaged to give a figure for the per cent antagonism of histamine at each dose level of benadryl.

On plotting, for a given experiment, the per cent inhibition of histamine against the effective dose of benadryl, it was noted that in all animals the dose-effect curve assumed the shape of a rectangular hyperbola with inhibition rising sharply at low doses of benadryl and then at a dose of about 1.0 mg. per kilo rapidly approaching an asymptote at about 97 per cent inhibition (fig. 5).

In view of the previously mentioned possibility that benadryl may act by combining with the site of action of histamine, disturbing, thereby, a histamine equilibrium, it was thought that benadryl might be adsorbed onto the effector site and thus the dose-effect relationships for benadryl should follow known adsorption patterns.

By assuming that the per cent inhibition of histamine at any effective concentration of benadryl is a measure of the amount of benadryl which is adsorbed onto the site of action of histamine, it was then possible to determine whether the dose-effect relationships for benadryl can be described by a known adsorption equation.

The adsorption equation which was chosen was the Langmuir isotherm (4)

$$\frac{C}{\alpha} = \frac{1}{\alpha\beta} + \frac{C}{\beta}$$

which states that the ratio of material to be adsorbed (C), in the present instance the effective concentration of benadryl in mg. per kilo, to adsorbed material (a), in the present instance the per cent inhibition of histamine, is a linear function of the material to be adsorbed (C). Or, in other words, if the present data are compatible with an adsorption phenomenon, then the ratio of the concentration of benadryl to the per cent inhibition of histamine should be a linear function of the concentration of benadryl.
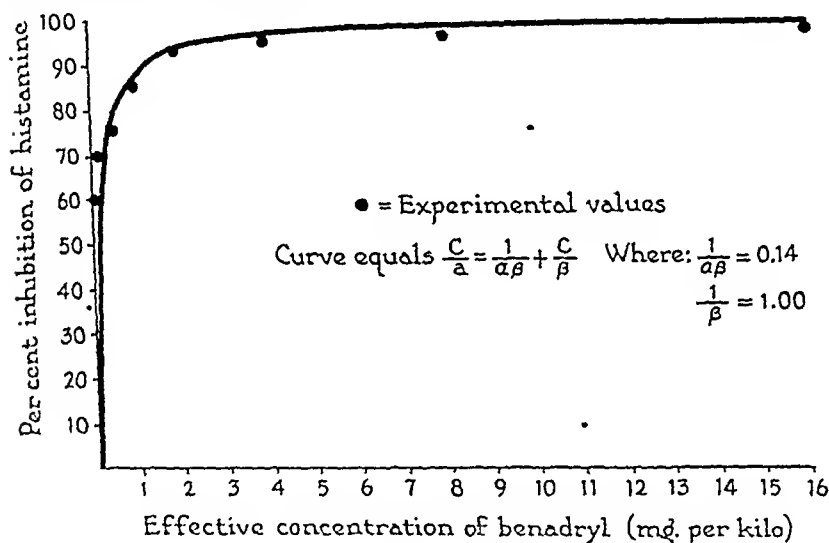
Fig. 6

A plot of these values for each experiment in this study showed this to be the case. From such a linear relationship slope $\left(\frac{1}{\beta}\right)$ and intercept $\left(\frac{1}{\alpha\beta}\right)$ constants can be determined, by means of which it is possible to fit the experimental data obtained with the Langmuir equation. An example of the adequateness of the fit can be seen on one of these experiments (fig. 6).

It may then be concluded, as to the magnitude of the antagonism of histamine by benadryl, relative to the effects on the dog's blood pressure, that a practical maximum inhibition of about 94 per cent is obtained with 4.0 mg. per kilo of benadryl. It may be concluded, as to the mechanism of this antagonism, that benadryl, by being adsorbed onto the site of action of histamine may so disturb the histamine equilibrium that a given amount of histamine has much less oppor-

tunity to reach and combine with its site of action.   Such an antagonism can be thought of as competitive.

DISCUSSION

The mode of action which has been suggested for benadryl in the present paper is perhaps best thought of as a competition between histamine and benadryl for a given site of action or receptive substance.   It is implied that if benadryl combines with the receptive substance no particular reaction occurs, but that it prevents, thereby, histamine from combining with this same site with the production of a biological effect.

Such a mode of drug antagonism is, of course, not novel.   It has been well demonstrated for other types of antagonism such as the atropine-acetyl-choline system (5), the para aminobenzoic acid-sulfonamide system (6), and the ergotoxine-epinephrine system (7).

More complete consideration of these mechanisms is to be found in the work of Clark (8) and in a recent discussion by Gaddum (9).

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ANTITHYROID STUDIES

III. The Goitrogenic Activity of Certain Chemotherapeutically Active Sulfones and Related Compounds

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The investigations of MacKenzie and MacKenzie (1) and of Astwood and his coworkers (2, 3) have shown that many of the chemotherapeutically active sulfonamides were goitrogenic. Since this laboratory has been studying the chemotherapeutic value of sulfones which are, in effect, rearrangement products of sulfonamides, these compounds and some of the corresponding sulfides have been tested for their antithyroid effect in rats. This investigation was stimulated by the observation of Higgins and Larson (4) that 4-aminophenyl 2'-amino-5'-thiazolyl sulfone (Promizole) caused marked thyroid hyperplasia in young rats when administered for several weeks. The heterocyclic substituted sulfones and sulfides which have been tested are listed in table 1. These were supplied by Dr. L. L. Bambas who has described their synthesis elsewhere (5).

Pohl (6) has shown that administration of thiourea to animals caused the appearance of an odorous substance in the expired air. On the basis of his experiments he concluded that the odor was due to exhaled methyl on ethyl sulfide. It is conceivable that these substances could be easily oxidized to the corresponding sulfone and partially excreted as such. For this reason we have investigated diethyl sulfone to establish its possible role in the antithyroid activity of thiourea.

Astwood demonstrated that 2-aminothiazole, a simple heterocyclic component of sulfathiazole, is an active goitrogen (3). In the first paper of this series, we have reported that thiazoline-2-thiol (4,5-dihydro-2-mercaptothiazole) was more active than thiouracil. Certain derivatives of this and other simple heterocyclic compounds have therefore been tested for antithyroid activity. It may be noted that some of the heterocyclic ring compounds shown in table 3 constitute the heterocyclic component of the sulfones listed in table 1.

RESULTS. The method used to evaluate these compounds has been described (7).

Sulfones. As a group the sulfones included in table 1 are relatively inactive. In agreement with the results of Higgins and Larson (4), Promizole induced hyperplastic thyroids but only with large doses in our 10-day test, and is 18 per cent as active as thiouracil. It is, however, the most active of the group of sulfones tested. Diethyl sulfone is inactive and we conclude that if thiourea is converted into the body to diethyl sulfone, the activity of the former is not due to the formation of this sulfone.

Sulfides. If the active goitrogens inhibit thyroid hormone synthesis by interference with the oxidative mechanism in the thyroid gland, the sulfides cor-

TABLE 1

Sulfones
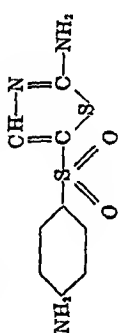
COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD	AVERAGE DOSE	BODY WT. GAIN	THYROID WT. MG./100 GM. RAT	THYROID IODINE	ESTIMATED ACTIVITY THIOURACIL = 100
			per cent	*mg/kg/day*	*gms/day*		*mg. %*	
Diethyl sulfone	$C_2H_5SO_2C_2H_5$	3 3	0.01 0.10	12 113	3.7 3.3	6.7 6.7	71.2 59.7	0
Ethyl phenyl sulfone	$C_6H_5SO_2C_2H_5$	3 3	0.01 0.10	11 109	3.3 3.0	6.5 8.2	51.4 51.7	0
Phenyl phenacyl sulfone	$C_6H_5SO_2CH_2COC_6H_5$	3 3	0.01 0.10	11 108	3.4 3.5	5.8 7.1	51.8 68.4	0
4,4'-Diaminodiphenyl sulfone	$NH_2C_6H_4SO_2C_6H_4NH_2$	3 3	0.01 0.10	12 106	3.9 1.5	6.8 10.0	63.3 18.3	4
4,4'-Diazoimidodiphenyl sulfone	$N_2C_6H_3SO_2C_6H_4N_2$	3 3	0.01 0.10	13 113	3.8 3.2	7.9 7.8	68.3 44.8	0
4-Aminophenyl 4'-n-propylamino-phenyl sulfone	$NH_2C_6H_3SO_2C_6H_4NHC_3H_7$	6 6	0.01 0.10	12 112	3.4 3.0	7.1 8.3	30.2 26.5	14
4-Aminophenyl 2'-amino-5'-thia-zolyl sulfone. (Promizole)		3 3 3 6 3 3 6 3 3 3	0.001 0.003 0.006 0.01 0.03 0.06 0.10 0.30 0.60 1.0	1.2 3.4 7.1 13 36 75 109 300 650 1000	4.1 3.5 3.6 3.7 3.8 3.8 2.0 1.8 0.4 1.0	7.6 8.5 8.0 9.6 11.3 24.4 20.0 34.2 40.7 18.6	43.5 28.4 28.8 27.2 9.3 2.0 2.1 2.8 1.8 9.8*	18
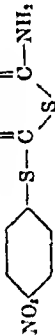
TABLE 1 (Continued)

COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD (per cent)	AVERAGE DOSE (mg./kt./day)	BODY WT. GAIN (gms./day)	THYROID WT. GAIN (gms./day)	THYROID IODINE (mg. %)	ESTIMATED ACTIVITY THIOURACIL = 100
4-Aminophenyl-2'-acetaminn-5'-thiazolyl sulfone	$NH_2C_6H_4SO_2C=CN=C(NHCOCH_3)S$	3 / 3	0.01 / 0.10	12 / 114	3.3 / 3.8	8.3 / 15.4	33.0 / 6.1	9
4-Aminophenyl-2'-amino-4'-methyl-5'-thiazolyl sulfone	$NH_2C_6H_4SO_2C=C(CH_3)N=C(NH_2)S$	3 / 3	0.01 / 0.10	11 / 107	3.2 / 3.0	7.4 / 20.8	30.6 / 4.0	14
4-Aminophenyl-2'-allylamino-4'-methyl-5'-thiazolyl sulfone	$NH_2C_6H_4SO_2C=C(CH_3)N=C(NHC_3H_5)S$	3 / 3	0.01 / 0.10	12 / 81	2.3 / 0.0	7.4 / 9.0	50.0 / 39.3	1
4-Aminophenyl 2'-nitrothienyl sulfone	$NH_2C_6H_4SO_2C=CHCH=C(NO_2)S$	3 / 3	0.01 / 0.10	10 / 107	2.8 / 2.9	0.8 / 7.3	80.0 / 25.9	2
4-Aminophenyl 2'-amino-5'-thia-diazolyl sulfone	$NH_2C_6H_4SO_2C \underset{S}{\overset{N\!-\!N}{\|}} C\!-\!NH_2$	3 / 3	0.01 / 0.10	11 / 120	3.6 / 4.1	8.4 / 8.3	43.0 / 35.0	1
Ethyl sulfonylacetyl urea	$C_2H_5SO_2CH_2CONHCONH_2$	3 / 3	0.01 / 0.10	12 / 102	3.0 / 2.7	0.4 / 5.0	53.0 / 63.0	0
n-Amyl sulfonylacetyl urea	$n\text{-}C_5H_{11}SO_2CH_2CONHCONH_2$	3 / 3	0.01 / 0.10	12 / 105	3.9 / 2.8	5.9 / 6.8	56.1 / 56.0	0
p-Methylsulfonylbenzamidine hydro-chloride	$CH_3SO_2C_6H_4C(=NH)NH_2 \cdot HCl$	3 / 3	0.05 / 0.3	58 / 282	3.5 / 0.6	0.0 / 5.7	57.5 / 77.5	0

* Two rats dead by seventh day not included in this data.

TABLE 2

Sulfides

COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD	AVERAGE DOSE	BODY WT. GAIN	THYROID WT. GAIN	THYROID IODINE	ESTIMATED ACTIVITY THIOURACIL = 100
			per cent	mg/kg/day	gms/day	gms/day	mg. per cent	
4,4'Diaminodiphenyl sulfide	(4-NH₂C₆H₄)₂S	3	0.01	10	2.5	8.4	32.1	15
		3	0.10	89	1.8	22.0	18.2	
1-Aminophenyl 2'-amino-5'-thiazolyl sulfide		3	0.0006	0.7	3.3	5.1	40.3	53
		3	0.001	1.2	3.3	5.9	45.7	
		3	0.003	3.6	3.4	7.7	35.2	
		3	0.006	6.6	3.3	7.8	22.1	
		9	0.01	11	3.1	9.3	11.3	
		3	0.03	35	2.7	15.5	2.1	
		6	0.055	55	2.6	23.7	1.7	
		9	0.10	96	2.4	28.0	1.8	
		3	0.30	284	1.4	38.2	1.4	
		3	0.5	308	1.0	29.9	1.9	
		3	1.0	532	−0.3*	23.3	4.6	
4-Aminophenyl 2'-acetamino-5'-thiazolyl sulfide		3	0.0006	0.7	3.2	0.2	73.3	17
		3	0.001	1.2	3.0	7.1	76.3	
		3	0.003	3.4	2.9	7.2	57.8	
		3	0.006	7.4	3.6	6.0	36.9	
		9	0.01	12	3.2	7.2	29.6	
		3	0.03	36	3.5	10.8	11.8	
		6	0.055	63	3.1	14.6	3.4	
		9	0.10	111	3.0	28.2	1.8	
		3	0.3	229	1.5	27.5	1.0	
		3	0.50	345	0.2	27.5	4.3	

4-Nitrophenyl 2'-amino-5'-thiadia-zolyl sulfide	$NO_2-\!\!\bigcirc\!\!-S-C\!\!\underset{S}{\overset{N=N}{\diagdown\!\!/}}\!\!C-NH_2$	6	0.01	12	3.5	9.3	16.7	38
		3	0.05	47	1.8	17.2	6.0	
		6	0.10	95	2.4	25.0	2.8	
		3	0.50	368	0.5	35.8	2.1	
		3	1.0	523	-0.9	26.2	4.0	
4-Aminophenyl 2'-amino-5'-thiadia-zolyl sulfide	$4\text{-}NH_2C_6H_4-S-C=N-N=C(NH_2)-S$	3	0.01	11	2.4	7.0	43.2	0
		3	0.10	107	1.4	26.3	3.4	

* One rat dead on sixth day of treatment.

responding to the above chemotherapeutically active sulfones should be more potent goitrogens because they are easily oxidized.   Comparison of table 1 with table 2 shows that the sulfides do possess markedly greater antithyroid properties. This fact is illustrated in the case of Promizole and its corresponding sulfide
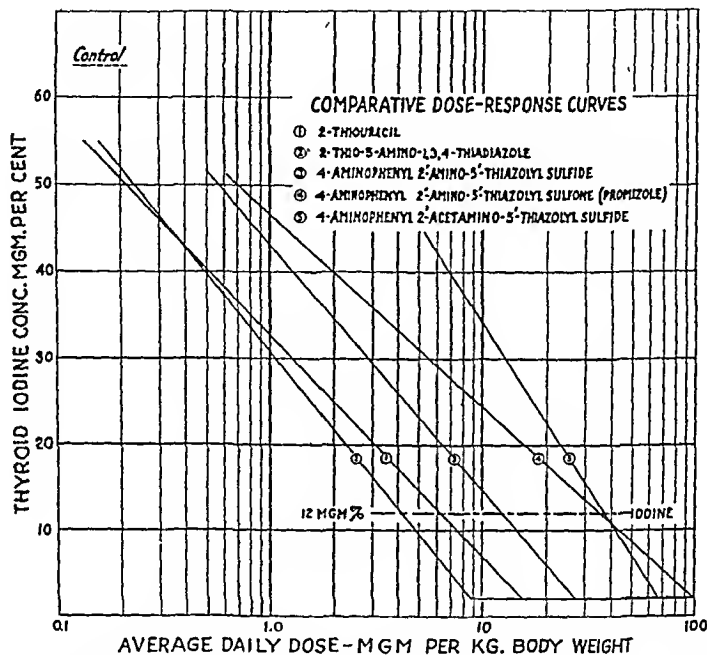
Fig. 1. Curves showing relationship between thyroid iodine concentration and dose for thiouracil, 2-thio-5-amino-1,3,4-thiadiazole, 4-aminophenyl 2'-amino-5'-thiazolyl sulfide, the corresponding sulfone and 4-aminophenyl 2'-acetamino-5'-thiazolyl sulfide. The compounds were given to rats in their food for a period of ten days.   The average daily dose was calculated from the concentration in the food and the average food intake.   The lines were calculated by the method of least squares from the experimental data   Activity was estimated from the dose under the intercept of the slope line and the 12 mgm. per cent base line, thiouracil being assigned a value of 100

(4-aminophenyl 2'-amino-5'-thiazolyl sulfide) by the dose-response curves which are recorded in figure 1 together with that of thiouracil.

Promizole
(18)

"Promizole Sulfide"
(53)
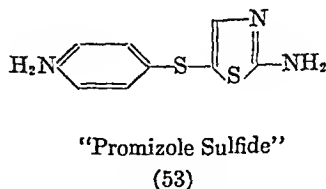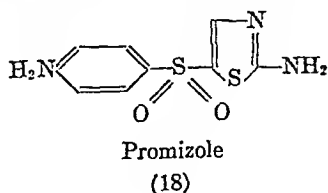
TABLE 3
Thiazolines, oxazolidines and thiadiazoles

COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD	AVERAGE DOSE	BODY WT. GAIN	THYROID WT. GAIN	THYROID IODINE	ESTIMATED ACTIVITY THIOURACIL = 100
			per cent	mg./kg./day	gms./day	gms./day	mg. per cent	
5-allyl-2-thiazoline-2-thiol	$CH_2CH_2N=C(SC_3H_5)S$	3	0.006†	11	3.2	6.0	39.2	8
		3	0.06†	57*	0.8	10.4	19.8	
2-Butylthio-5-methyl-2-thiazoline	$CH_3CHCH_2N=C(SC_4H_9)S$	3	0.006†	10	1.9	6.2	56.7	0
		3	0.06†	81	3.2	6.2	47.8	
5-Phenyl-2-thiazoline-2-thiol	$C_6H_5CHCH_2N=C(SH)S$	3	0.01	10	2.9	8.7	40.8	2
		3	0.10	112	3.2	6.9	24.9	
3-Butylthiazolidine-2-thione	$CH_2CH_2N(C_4H_9)C=SS$	3	0.006†	11	3.2	6.3	38.2	0
		3	0.06†	80	1.5	7.9	53.2	
3-Phenylthiazolidine-2-thione	$CH_2CH_2N(C_6H_5)C=SS$	3	0.01	11	3.3	5.4	60.6	0
		3	0.10	100	2.8	5.7	55.9	
1 Oxothiazolidine-2-thione (Rhodanine)	$COCH_2NHC=SS$	3	0.01	11	3.0	6.0	61.2	1
		3	0.10	107	3.1	7.2	32.7	
2,4-Dioxothiazolidine	$COCH_2N=COS$	3	0.01	12	3.0	5.9	38.9	6
		3	0.10	93	2.6	9.4	16.1	

TABLE 3—Continued

COMPOUND	FORMULA	NUMBER OF ANIMALS	CONCENTRATION IN FOOD	AVERAGE DOSE	BODY WT. GAIN	THYROID WT. GAIN	THYROID IODINE	ESTIMATED ACTIVITY THIOURACIL = 100
3-Phenyl-4-oxothiazolidine-2-thiono (3-Phenylrhodanine)	$COCH_2N(C_6H_5)C{=}SS$	3	0.01	12	4.1	5.5	65.4	0
		3	0.10	100	2.4	8.0	41.7	
3-Sulfomethyl-2-thio-2,4(3,5)-oxazolediono	$COCH_2OCSN{-}CH_2SO_3H$	3	0.01	13	4.1	6.5	49.0	1
		3	0.10	119	3.8	6.0	34.1	
3-β-Sulfoethyl-2-thio-2,4(3,5)-oxazolediono	$COCH_2OC({=}S)N{-}CH_2CH_2SO_3H$	3	0.01	12	3.1	9.1	66.1	0
		3	0.10	119	3.7	8.2	61.6	
3-p-Sulfonophenyl-2-thio-2,4(3,5)-oxazolediono	$COCH_2OC({=}S)NC_6H_4SO_3H$	3	0.01	12	3.9	7.5	40.5	0
		3	0.10	113	3.6	6.8	50.0	
5-Amino-1,3,4-thiodiazole-2-thiol	$H_2N{-}C\underset{\underset{S}{\diagdown}}{\overset{N{-}N}{\diagup}}C{-}SH$	6	0.0002	0.2	3.4	7.3	52.1	156
		6	0.0003	0.4	3.3	6.5	41.1	
		3	0.0004	0.5	3.4	6.9	44.2	
		6	0.00055	0.7	3.7	7.0	31.2	
		3	0.0007	0.9	4.4	6.8	27.6	
		6	0.001	1.2	3.2	8.7	27.6	
		3	0.0015	1.8	3.5	8.9	24.3	
		3	0.0025	3.0	3.6	8.4	8.1	
		3	0.003	3.9	3.9	0.7	7.3	
		6	0.01	11.0	3.0	16.4	6.3	
		3	0.03	38.0	3.7	29.6	1.3	
		3	0.06	67.0	3.0	26.4	2.4	
		6	0.10	100	2.8	28.5	2.0	
		3	0.30	358	2.5	30.5	1.4	
		3	0.60	550	0.9	37.2	2.6	
		3	1.0	910	-0.3	17.0	5.7	

		per cent	sm./kg./day	gms./day	gms./day	mg. per cent	
1,3,4-Thiadiazole-2,5-dithiol	$\overset{S}{HSC=N—N=CSH}$	6 / 3	0.01 / 0.10	12 / 111	3.8 / 4.0	6.9 / 7.7	39.3 / 28.5	7
N,N'-Dibutyl-2,2'-dithio-bis-ethylamine	$C_4H_9NHCH_2CH_2—S—S—CH_2CH_2NHC_4H_9$	3 / 3	0.006† / 0.06†	10 / 75	3.4 / 2.7	6.8 / 7.9	63.1 / 32.2	2
p-Aminomethylbenzenesulfon-amide (Marfanil)	$NH_2SO_2C_6H_4CH_2NH_2$	3 / 3	0.05 / 0.50	54 / 590	1.9 / 3.6	8.5 / 6.8	41.0 / 65.3	0
Di-(p-sulfamylbenzyl) amino	$(NH_2SO_2C_6H_4CH_2)_2NH$	3 / 3	0.05 / 0.3	57 / 370	3.6 / 2.6	6.6 / 7.2	44.5 / 50.4	0
p-Hydroxybenzylamino hydro-chloride	$HOC_6H_4CH_2NH_2·HCl$	3 / 3	0.01 / 0.10	11 / 110	3.9 / 3.0	8.0 / 10.4	80.6 / 76.8	0
p-Hydroxybenzyl methyl amino hydrochloride	$HOC_6H_4CH_2NHCH_3·HCl$	3 / 3	0.01 / 0.10	13 / 123	4.1 / 3.5	8.2 / 9.5	79.0 / 72.3	0

* One rat dead on seventh day of treatment.
† Compound administered in the drinking water.

Acetylation of the above sulfide (curve 5) resulted in decreased activity, the compound having the same potency as Promizole. The slope of the line for Promizole diverges considerably from the average slope obtained for other compounds which we have studied (7, 8). While the reason for this discrepancy is not entirely clear, it is probable that this compound is metabolized differently than other sulfones.

Thiazolines, oxazolidines and thiadiazoles. Alkylation of 2-thiazoline-2-thiol led to decreased antithyroid potency (table 3). Oxidation of the parent compound to rhodanine (4-oxothiazoline-2-thiol) also resulted in decreased activity, but conversion of rhodanine to 2,4-dioxothiazolidine caused an increase in activity as judged by the limited experimental data of screening tests. Introduction of a phenyl group into rhodanine had little effect on its antithyroid

<div align="center">

TABLE 4

2-Aminothiadiazoles

$$N_3\text{———}N_4$$
$$R\text{—}C_2 \quad {}^5C\text{—}NH_2$$
$$\underset{S}{\diagdown}$$

</div>

R	ACTIVITY (THIOURACIL = 100)
NH₂⟨ ⟩—SO₂—	6
NH₂⟨ ⟩—S—	9
NO₂⟨ ⟩—S—	38
HS—	156

activity. In view of these results further investigation of simple alkyl and aryl derivatives of 2-thiazoline-2-thiol has been discontinued. Three oxazolidines containing a sulfonic acid grouping were tested and found to be inactive. Oxazolidine-2-thiol itself has not been studied in our laboratory.

One of the most actively antithyroid compounds found in this series was 5-amino-1,3,4-thiadiazole-2-thiol (TC-68). By reference to tables 1 and 2 it will be seen that it is the heterocyclic component of a p-aminophenyl sulfide and sulfone, neither of which possess pronounced goitrogenic properties. Comparative activity of these compounds as well as that of one p-nitro-derivative, is recorded in table 4. It is noteworthy that as the substituent becomes less complex, the antithyroid activity increases. This observation is in conformity with that made in other series where it has been shown that substitution of hydrocarbon groups in the simple heterocycle destroys activity. Replacement of the 2-amino group by an —SH group resulted in greatly decreased potency.

TC-68 is relatively non-toxic in doses as high as 350 mgm. per kg. per day for the 10 day test period. In view of this relative non-toxicity and its high potency, TC-68 is to be studied in detail.

Marfanil (p-aminomethyl benzene sulfonamide) and the corresponding disubstituted compound [di-(p-sulfamylbenzyl)amine] are both inactive goitrogens even when administered in 0.5 and 0.3 per cent concentration in the food respectively. Rats fed either substance drank considerably more water than untreated rats and in the case of marfanil at the higher dose level, had diarrhea. The cause of this is unknown. When the sulfamyl grouping of marfanil is replaced by an hydroxyl (p-hydroxybenzyl amine) and the latter fed to rats, thyroid hyperplasia occurred but rather than a greatly diminished total thyroid iodine concentration as would be expected with any trace of goiter, the iodine concentration showed a slight increase. This same effect was noted in the case of p-hydroxybenzyl methyl amine. An explanation for this effect is not apparent.

SUMMARY

Thirty eight compounds including 14 chemotherapeutically active sulfones and sulfides, were tested for their antithyroid activity in rats. Promizole was the most active goitrogen of the sulfone group, having, however, only 18 per cent of the activity of thiouracil. In general, sulfides corresponding to the sulfones were more active, "Promizole sulfide" showing 53 per cent of the potency of thiouracil.

Of the simple heterocycles related to the above compounds only 5-amino-thiadiazole-2-thiol (TC-68) exhibited marked antithyroid activity and is being investigated further. Certain relationships between chemical structure and antithyroid activity of these simple heterocyclic compounds have been discussed in terms of the experimental data.

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STUDIES ON THE MECHANISM OF ACTION OF SYMPATHOMIMETIC AMINES

I. The Effect of Various Amines on the Synthesis of Cocarboxylase

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Considerable knowledge has been accumulated as to the mode of inactivation of sympathomimetic amines by tissues but relatively few papers have appeared which throw light on the mechanisms whereby these compounds are able to exert their well-known pharmacological "actions." If one selects one of these effects, namely the pressor phenomenon, one must conclude that a source of energy must be quickly activated to supply fuel for smooth muscle contraction. Since the energy-rich phosphate linkage is one of the largest rapidly available sources of potential energy in tissue oxidations, the authors thought it of possible interest to investigate the effect of pressor amines on phosphorylation mechanisms. The system here reported, namely that of cocarboxylase synthesis by liver tissue, was chosen since its mechanism has been well worked out and a specific method for cocarboxylase estimation exists, although at present there is no basis for believing that this system is implicated directly in the sympathomimetic effect.

METHODS. It was demonstrated by Ochoa (1) and by Govier and Greig (2) that pigeon liver slices (but not homogenate) can synthesize cocarboxylase from added thiamin and pyrophosphate, under aerobic conditions. This tissue was employed in the present study.

Free hand slices were cut from livers of normal pigeons and immediately placed in oxygenated Ringer-veronal buffer, pH 8.5. They were blotted lightly and placed in previously prepared vessels containing thiamin in final concentration of $100\gamma/cc.$, sodium pyrophosphate in 0.01 M final concentration, and Ringer-veronal buffer, pH 8.5 to make a final volume of 3.0 cc. Sympathomimetic amines were added to the vessels to make a final concentration of 5×10^{-5} M. The vessels were affixed to simple constant volume manometers, equilibrated with oxygen, and shaken for one hour at 37°. Each experiment contained a "start control", which was boiled at the time of placing the vessels in the bath, and a "run control" containing no amines, in addition to the vessels containing amines. After incubation for one hour the vessels were heated in boiling water for three minutes. The contents were homogenized, made up to a known volume, and centrifuged. The supernatant fluid was analyzed by allowing its cocarboxylase to combine with carboxylase apoenzyme prepared according to Green et al (3), and comparing the rate of pyruvate decarboxylation with that in vessels containing known quantities of cocarboxylase.[1] Increases in

[1] Kindly supplied by Merck and Co.

TABLE 1

NAME OF COMPOUND	5	4	3	2	C	C	N	RESULT: IN PERCENT-AGE CHANGE IN COCARBOXYLASE
Phenethylamine								+39.8 +22.7 +18.8 +10.4 −16.8 +15.1 average
N-methyl phenylethyl-amine							CH$_3$	+36.5 +28.6 +27.7 +21.0 +28.4 average
N-dimethyl-β-methyl-phenylethylamine					CH$_3$		(CH$_3$)$_2$	+18.0 +35.8 +16.1 +23.3 average
Amphetamine						CH$_3$		+12.6 +26.7 +35.9 +25.1 average
Tyramine		OH						+12.1 −26.0 +16.8 +54.6 +17.7 +15.0 average
β(p-hydroxyphenyl) iso-propylamine ('Paredrine')		OH				CH$_3$		+44.3 +45.4 −6.0 +14.9 +36.8 +27.1 average

The table header shows "FORMULA" with a ring structure numbered 5, 4, 3, 2 with −C−C−N substituent.

amount of phosphorylation were expressed in percentage by the following formula:

$$100 \times \frac{\text{Amount of cocarboxylase in presence of drug } - \text{ run control}}{\text{Amount of cocarboxylase in presence of drug } - \text{ start control}}$$

Decreases in amount of cocarboxylase, when they occurred, were calculated according to the following formula:

$$100 \times \frac{\text{run control } - \text{ cocarboxylase in presence of drug}}{\text{run control } - \text{ start control}}$$

The amounts of cocarboxylase were calculated as gammas of cocarboxylase per gram of wet tissue, prior to calculating the above percentages.   The mean values for start and run controls were 9.2 and 12.6γ of cocarboxylase per gram of wet tissue, respectively.

RESULTS AND DISCUSSION.   Table 1 contains the names and formulae of the amines which were tested, together with a summary of the results in which each figure represents a separate experiment and animal.   The results were calculated according to the above formulae.

It may be seen from the table that these compounds produce quite consistent increases in phosphorylation of thiamin.   When compounds having other types of substitution were tested in this system, the results obtained were quite variable. Some evidence has been obtained which leads us to believe that the oxidation products of the latter compounds are capable of inhibiting the carboxylase used in the cocarboxylase determination.   These data will be reported in the future. None of these compounds showed any effect when added to purified carboxylase without previous incubation with tissue.

Further work, concerning the effect of these compounds on succinoxidase and the relation of this effect to the phosphorylation of thiamin, is reported in the following paper.

SUMMARY

Various sympathomimetic amines are capable of increasing the synthesis of cocarboxylase by pigeon liver slices *in vitro*.

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STUDIES ON THE MECHANISM OF ACTION OF SYMPATHOMIMETIC AMINES

II. The Effect of Sympathomimetic Amines on the Succinoxidase System as Influenced by the Presence of α-tocopherol Phosphate

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In view of the fact that sympathomimetic amines have no direct effect on purified carboxylase, as stated in the previous paper, one is brought to the conclusion that the energy supply for the increase in phosphorylation of thiamin reported previously must come from an increased rate of metabolism of some substrate.

The literature concerning the effect of epinephrine on respiration of tissues has already been discussed in the review of De Meio (2) and will not be covered completely here, but a few interesting points should be mentioned. Most reports of *in vivo* tests have shown a stimulation of metabolism; whereas investigators have variously reported no effect, stimulation, or inhibition of tissue respiration following the addition of the drug *in vitro*. This variation has been attributed to pH differences (3) and to variation in oxygen tension (4). There is still another possible factor which may play a role in these variations. If one examines the literature he is impressed that the degree of dispersion of tissue is of some importance, and that inhibitory effects are more likely to appear in extracts, homogenates, and the like, whereas stimulatory effects are commoner *in vivo* and in whole cell preparations.

In consideration of these points, one may say in general that for epinephrine to produce a stimulatory effect on metabolism, it probably should function in a system which is already under physiological control from the standpoint of rapidity of oxidations. It seems reasonable that much less energy might be expended in changing the rate of oxidation of an already balanced system than would be necessary to accelerate a system which is metabolizing at maximal speed for the concentration of enzymes and substrates available.

An example of a normally inhibited system is that of muscle succinoxidase in the presence of adequate amounts of α-tocopherol phosphate as described by Houchin and Mattill (5). These workers have shown that α-tocopherol phosphate (α-TPh) is capable of reducing the high succinoxidase activity of Vitamin E-deficient muscle to normal values, but state that α-TPh has no effect on normal muscle succinoxidase. On the basis of this work we thought it of interest to ascertain whether or not sympathomimetic amines are capable of influencing a succinoxidase system which is held under physiological control by α-TPh.

The following experiments were designed to determine whether or not α-TPh can affect tissue from normal rats, and whether or not sympathomimetic amines can relieve tocopherol inhibition.

The effect of α-TPh[1] was investigated on a normal succinoxidase system of rat skeletal muscle as described by Schneider and Potter (6). The rats used were normal adult albino males of Wistar strain, maintained on a diet of 'Friskies' dog food. They were killed by decapitation, and the leg muscles homogenized in ten times their weight of M/15 phosphate buffer, pH 7.2. The experiments were carried out using simple constant volume manometers. Each vessel contained (together with varying concentrations of α-tocopherol phosphate) the following:

```
Tissue homogenate.....  .................... 0.5 cc.
Cytochrome C............................ 0.3 cc., containing 4 × 10⁻⁸ moles
AlCl₃ 0.012 M ⎫
             ⎬combined.....  .. ........... 0.1 cc.
CaCl₂ 0.012 M⎭
Sodium succinate M/2...................... 0.3 cc. in side bulb
KOH, 20%.......... ............... ....... 0.2 cc. in center well
Buffer, M/15 phosphate, pH 7.2, to make 3.0 cc.
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When α-TPh was added to such a system in concentrations varying from 0 to 5.8×10^{-3} M a progressive inhibition resulted as illustrated by the data plotted in figure 1.

Since these rats were maintained with a diet believed to be adequate in Vitamin E, we may conclude that α-TPh can inhibit a normal succinoxidase system. This finding would suggest that the α-tocopherol derivative present in the tissue before homogenization may diffuse out when the cells are ruptured, as does cytochrome C, thus making it necessary to add more tocopherol to insure physiologic saturation of the system. We have attempted to prove this point by the estimation of tocopherol by the method of Devlin and Mattill (7) in supernatants and centrifugates of centrifuged muscle homogenates. Although we were able to detect α-tocopherol in the supernatant fluid in considerable amounts after centrifugation, we have consistently been unable to extract as much tocopherol from an uncentrifuged homogenate as was present in the combined values for centrifugate and supernatant of an equal volume of centrifuged homogenate. This throws the absolute values in doubt. However, since tocopherol is present in the supernatant, one may logically conclude that it has diffused out of the cells and away from enzyme proteins.

When one adds varying quantities of epinephrine to a tocopherol inhibited succinoxidase system, the results vary according to the degree of tocopherol inhibition as well as the amount of epinephrine added. If epinephrine is added to an uninhibited rat muscle succinoxidase preparation, either no effect or a slight inhibition of oxygen uptake is seen. If α-TPh is added in amounts not sufficient to saturate the succinoxidase system, and epinephrine is added in

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[1] We are indebted to Dr. R. D. Shaner of Hoffman-LaRoche, Inc. and to Dr. E. M. Schultz of the Dept. of Organic Chemistry, Sharp and Dohme, Inc., for a supply of α-tocopherol phosphate.

smaller molar concentration than that of the tocopherol, the epinephrine produces variable results, sometimes stimulating, and sometimes inhibiting the system. If, however, α-TPh is added in a concentration sufficient to saturate the system (i.e. for maximal inhibition), and epinephrine concentrations of magnitude equal to or greater than that of the tocopherol are used, epinephrine and all other sympathomimetic amines tested (except 'Paredrine'[2], added as the hydrobromide) produce an increase in oxygen uptake.
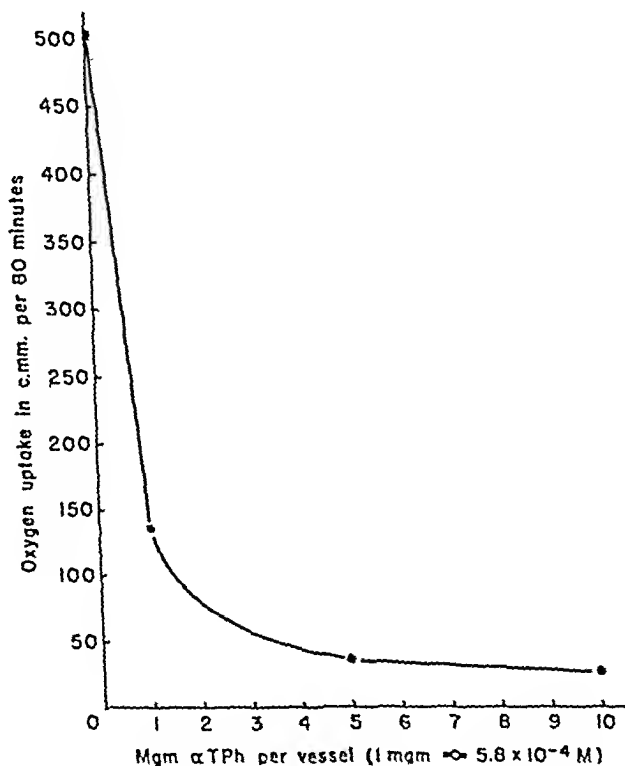
FIG. 1

The removal of tocopherol inhibition by amines may be illustrated by the following experiment:

Each vessel contained:

Tissue	0.5 cc. of a 10 per cent rat muscle homogenate
Cytochrome C	0.3 cc. containing 4×10^{-5} moles
AlCl₃	0.1 cc. of 12×10^{-3} M

[1] 'Paredrine' is the trade mark applied by Smith, Kline and French, Inc. to β(4 hydroxy phenyl) isopropylamine.

CaCl$_2$................ 0.1 cc. of 12 × 10^{-3} M
α-TPh............... 0 or 0.2 cc. (final concentration 5.9 × 10^{-4} M)
Amine............... 0 or 0.2 cc. of 0.018 M (1.2 × 10^{-3} M final concentration)
Sodium succinate.... 0.3 cc. of 0.5 M added from side bulb
KOH, 20%............ 0.2 cc. in center well
Buffer to make 3 cc. total volume (phosphate M/15, pH 7.2)

Concentrations:
αTPh: 5.9 × 10^{-4} M
amines: 1.2 × 10^{-3} M
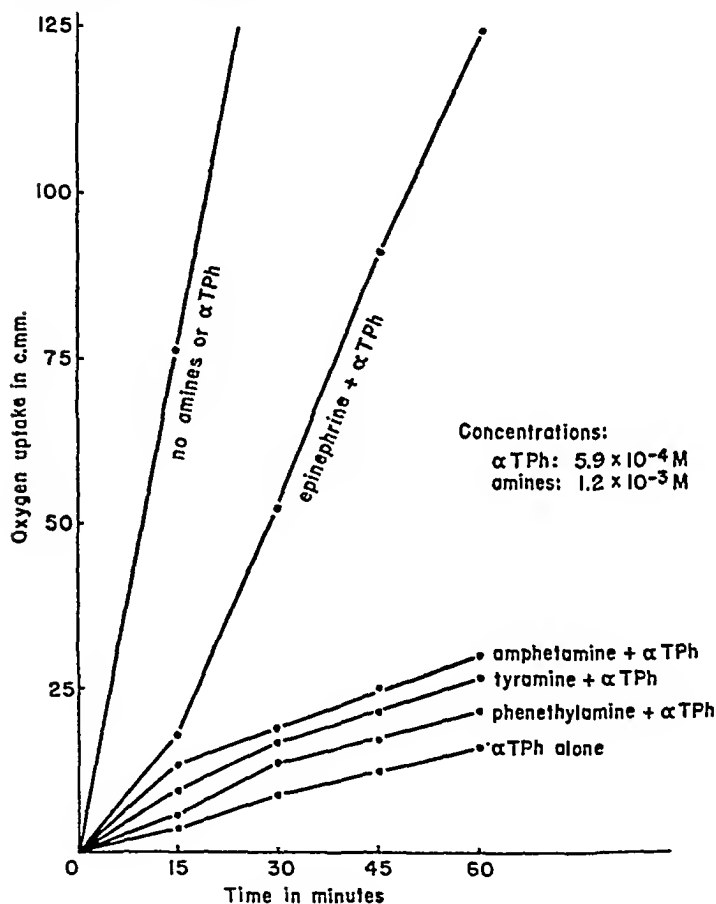
Fig. 2

The data plotted in figure 2 illustrate the increase in oxygen uptake of an α-TPh-inhibited succinoxidase system resulting from the addition of various sympathomimetic amines.

The addition of increasing concentrations of amines results in progressive acceleration of oxygen uptake until a concentration of about 6 × 10^{-2} M is

reached, after which inhibition occurs. Amphetamine, 'Cobefrin'², epinephrine, and phenethylamine have been used in this type of experiment. Figure 3 illustrates a typical experiment, using amphetamine.
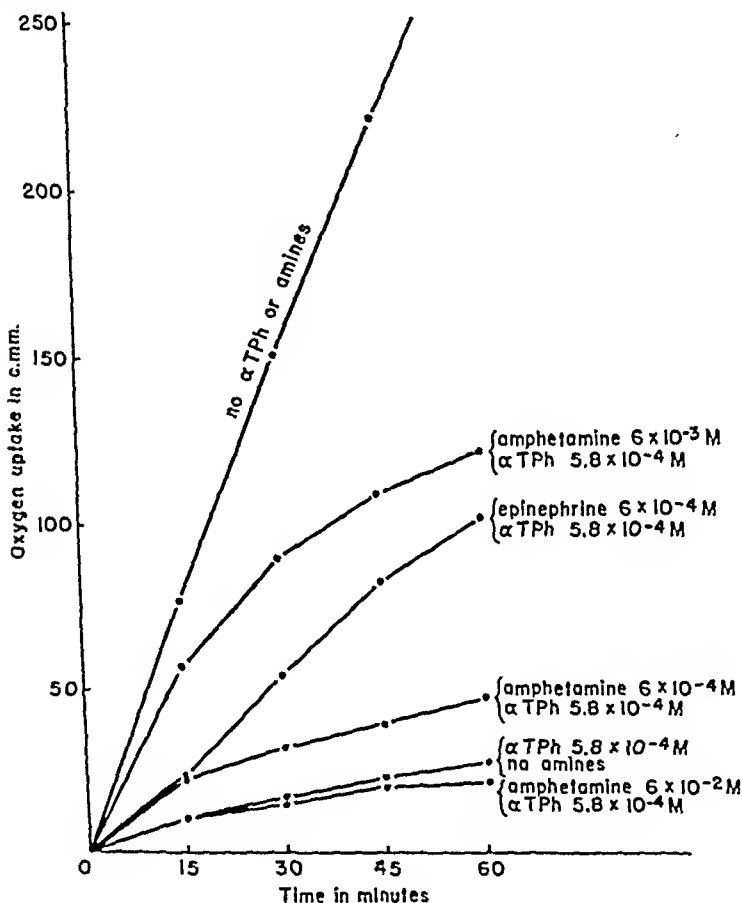
Fig. 3

The above experiments have indicated that α-tocopherol probably diffuses away from enzyme proteins during homogenization, that the addition of α-TPh to a succinoxidase system results in the lowering of the rate of oxygen uptake, and that the addition of sympathomimetic amines removes or lessens the tocopherol inhibition. The previous paper demonstrated that cocarboxylase

² 'Cobefrin' is the trade mark applied by Winthrop Chemical Co. to β(3,4 dihydroxyphenyl) β-hydroxyisopropylamine.

synthesis is accelerated by the addition of amines to pigeon liver slices. It is known (8) that homogenates are usually unable to synthesize cocarboxylase. We thought it of interest to determine whether or not homogenates fortified with cytochrome C could phosphorylate thiamin and to determine the effect of the addition of amines with and without tocopherol on such synthesis.

The problem of the phosphorylation of thiamin in the presence of a succinoxidase system was attacked in the following manner:

A succinoxidase system was set up in a similar manner to that described above, using homogenized rat liver as a source of succinoxidase, and containing added thiamin and pyrophosphate as described in the previous paper. Amphetamine was selected as the sympathomimetic drug since it is not oxidized by tissue. The results of four experiments are given in table 1, in which cocarboxylase is expressed in γ/gram of wet tissue after one hour's incubation at 37° in an atmosphere of air.

It may be seen from table 1 that phosphorylation does occur in a tissue homogenate which contains added cytochrome C and that it occurs from energy

TABLE 1

EXPER. NO.	COCARBOXYLASE (γ PER GRAM OF WET TISSUE)					O_2 UPTAKE IN C. MM /HR.	
	Start control	Run control	Amphetamine added	α-TPh added	Amphetamine and α-TPh added	Amphetamine added	Amphetamine and α TPh added
1	12.7	11.7	16.0	14.4	14.7	960.0	138.0
2	6.0	6.3	10.7	10.5	11.5	820.0	111.4
3	12.0	12.6	11.7	11.4	12.9	753.0	111.3
4	10.1	11.1	12.9	11.5	14.3	751.5	82.0

supplied by succinate oxidation, since the latter substrate is in great excess. The addition of amines accelerates the phosphorylation as it did with tissue slices (1) (although not to the same magnitude) and in most cases the combination of α-TPh and amine produced the highest value for cocarboxylase. In view of the fact that tissues in the presence of a combination of α-TPh and amine have a much lower rate of oxygen uptake than do those to which α-TPh has not been added, these results would suggest that thiamin synthesis is more efficient in the presence of α-TPh than in its absence, if the efficiency is calculated on the basis of the amount of oxygen used per gamma of cocarboxylase formed.

A final point should be mentioned. We have found that sympathomimetic amines are not the only compounds capable of relieving tocopherol inhibition. Ascorbic acid and cysteine have a like function, but these are normal tissue components and should be present *in vivo* in reasonably constant amounts, whereas sympathomimetic amines are present either intermittently, or in the case of epinephrine or sympathin, in suddenly varying concentrations. It is interesting to note that cystine inhibits succinoxidase which is not already depressed by α-tocopherol (9). Ergotamine and acetylcholine have no effect in this system.

We believe that the fact that sympathomimetic amines can produce increased metabolic activity in a succinoxidase system by reversing the normal inhibitory effect of α-tocopherol offers at least one explanation for an increased energy production when sympathomimetic amines are added to homogenized tissue preparations. Further work is being continued in an effort to extend these findings to whole cell preparations.

SUMMARY

1. Succinoxidase of homogenized rat muscle and liver is inhibited by added α-tocopherol phosphate. This effect may be due to diffusion away from the succinoxidase of α-tocopherol originally present in the tissue, upon rupture of the cells during homogenization.

2. Sympathomimetic amines are capable of producing an increase in oxygen uptake by relieving the tocopherol effect.

3. The increase in metabolic activity following the addition of amines to a tocopherol-inhibited succinoxidase system can be used as a source of energy for the synthesis of cocarboxylase from added thiamin and pyrophosphate.

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STUDIES ON SHOCK INDUCED BY HEMORRHAGE

XIII. Isolation of a Lactic Dehydrogenase Inhibitor from Liver[1]

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An earlier paper in this series reported that anoxia decreased the ability of rat and pigeon liver to metabolise lactic and amino acids (1). Since the change in tissue coenzyme content under the conditions of our experiments did not account for the decreased metabolic activity, we assumed that some inhibitor, liberated or made active by anoxia, might be responsible. It was found that liver extract did contain a thermolabile globulin-like substance which inhibited the oxidation of amino acids in whole tissue, as well as their oxidation by isolated *d*-amino acid oxidase. In the latter case it was shown that the inhibitor was most active at low pH's and in low concentrations of phosphate and that it formed a reversible combination with the apoenzyme-coenzyme-substrate complex of the amino acid oxidase system (2).

The addition of liver extracts to the lactic dehydrogenase system also produces a decrease in the rate of oxidation of lactate. This, however, can be partially accounted for by destruction of cozymase by an enzyme, nucleotidase, present in liver (3). In a previous communication decreases in O_2 consumption produced on addition of crude liver extract to lactic dehydrogenase and which were attributed to apoenzyme destruction were probably a summation of effects produced by cozymase hydrolysis and inhibition by the factor described here. When the nucleotidase is inhibited by nicotinamide (3) or when it is removed by partial purification of the extract, there remains an inhibitor which does not affect the coenzyme, and appears to be similar to, but not identical with, the inhibitor of *d*-amino acid oxidase.

EXPERIMENTAL. *Methods.* Materials required were prepared as follows: lactic dehydrogenase by the method of Green and Brosteaux (4); flavoprotein by the method of Straub (5); and cozymase by that of Williamson and Green (6). Cozymase was determined according to Axelrod and Elvehjem (7) as already described (8). The extracts which contained inhibitor were aqueous suspensions of rat, dog, or beef livers, which in some experiments were partially purified by precipitation with ammonium sulphate as previously described (2). Adsorption on and elution from $Cu(OH)_2$ gave products which were sometimes active.

Oxygen consumption was measured in Warburg manometers which usually contained 0.5 cc. enzyme, 0.75 cc. flavoprotein solution; 0.2 cc. cozymase (300γ); 0.2 cc. extract (boiled or active); 0.1 cc. nicotinamide (50%); 0.2 cc. lactate (2M); 0.25 cc. sodium cyanide (M), adjusted to pH 7; 0.1 cc. Methylene blus (.5%). No KOH papers were used as the oxidation of lactate to pyruvate involves only oxygen consumption and no CO_2 evolution.

RESULTS. *Prevention of nucleotidase action on cozymase.* Mann and Quastel (3) have shown that the hydrolysis of cozymase by nucleotidase may be prevented by nicotinamide. Table I shows that under the conditions of our experiments this hydrolysis is completely inhibited by a final concentration of 2% nicotinamide. When cozymase was incubated with active extracts large decreases occurred due to the nucleotidase present in the extracts. Boiling inactivated the nucleotidase and prevented cozymase destruction. When nicotinamide was added to active extracts prior to incubation, the cozymase levels were the same as after incubation with boiled extract, indicating that nucleotidase activity was completely inhibited.

Effect of liver extract on lactic dehydrogenase. Table 2 shows the effects of crude and partially purified extracts on the isolated lactic dehydrogenase system. In all cases nicotinamide was present to prevent hydrolysis of cozymase by the

TABLE 1

Analyses for cozymase after incubation with liver extracts (crude and partially purified) with and without nicotinamide

Tubes containing 0.2 cc. extract, 0.1 cc. cozymase, 0.033 cc. nicotinamide (50%), 0.5 cc. buffer or NaCl solution were incubated at 37°C. for 45–60 min., heated to destroy extract and analysed for cozymase.

EXPERIMENT	EXTRACT	COZYMASE (γ)		
		Boiled ext.	Active ext. + nicotinamide	Active ext. without nicotinamide
232	Crude rat liver	188	182	39
246	Crude rat liver	119	118	0
80	Beef liver; $(NH_4)_2SO_4$ ppt.	70	86	85
87	Beef liver; $(NH_4)_2SO_4$ ppt.	100	100	100
105	Beef liver; $(NH_4)_2SO_4$ ppt.	113	112	118

nucleotidase. It will be seen that the crude extracts usually contain considerable amounts of the lactic dehydrogenase enzyme since the rate of oxidation of lactate is considerable with extract and boiled enzyme (column 3). This value may justifiably be subtracted from the value obtained for active extract and active enzyme so long as the limiting factor is the apoenzyme, however, it will be seen that the value obtained for active extract and enzyme is usually lower than that for boiled extract and enzyme, thus indicating some inhibition, even without correcting for lactic dehydrogenase in the extract.

When the partially purified extracts which contain very little lactic dehydrogenase are used, the decrease in O_2 consumption produced by inhibitor is not masked by the addition of apoenzyme contained in the extracts, nor is nucleotidase present. In these instances the small values obtained for the active extract and boiled enzyme are subtracted from the values for active extract and active enzyme before calculating the percentage of inhibition. It may be seen that inhibitions of around 30% are produced.

Evidence that the inhibitors of d-amino acid oxidase and lactic dehydrogenase are different. If the inhibitors found in liver extract are identical for these two

TABLE 2

The effect of liver extracts on the activity of lactic dehydrogenase

Nicotinamide was present in all the experiments to prevent possible hydrolysis of cozymase by nucleotidase.

EXP.	EXTRACT	MM³ O₂ CONSUMED			% INHIBITION
		Enzyme +		Boiled enzyme + active ext.	
		boiled ext.	active ext.		
214	Crude rat liver	270	233	144	
229	Crude rat liver	554	427	283	
252	Crude rat liver	270	233	55	
75	Crude rat liver	414	362	225	
275	Dog liver; (NH₄)₂SO₄ ppt.	210	192	44	29
75	Beef liver; (NH₄)₂SO₄ ppt.	456	315	17	34
81	Beef liver; (NH₄)₂SO₄ ppt.	294	220		25
86	Beef liver; (NH₄)₂SO₄ ppt.	154	112		27

TABLE 3

Comparison of effects of various extracts on d-amino acid oxidase and lactic dehydrogenase

EXPERIMENT	EXTRACT		PROTEIN N MO/VESSEL	% INHIBITION OF	
				d-amino acid oxidase	lactic dehydrogenase
100	94 II	Cu(OH)₂ eluate	0.0639	43	0
104	94 II	Cu(OH)₂ eluate	0.0319	35	0
107	87 IV	(NH₄)₂SO₄ ppt.	0.192	32	29
110	109 I	(NH₄)₂SO₄ ppt.	0.299	32	45
115	109 III	(NH₄)₂SO₄ ppt.		62	56
143	138	Cu(OH)₂ eluate	0.0429	29	38
	141	(NH₄)₂SO₄ ppt.	0.188	29	0
166	158 A	Cu(OH)₂ eluate I		43	50
162	158	Cu(OH)₂ eluate II		40	44
184	158 B III	(NH₄)₂SO₄ ppt.		26	52
170	158 B IV	(NH₄)₂SO₄ ppt.		9	9
202	183 I	(NH₄)₂SO₄ ppt.		39	0

enzymes, one would expect that if an inhibition were produced by an extract with one enzyme it would likewise be produced with the other enzyme. This, however, does not always occur. It will be seen in table 3 that a considerable inhibition of d-amino acid oxidase may be produced by extracts which have

no effect on lactic dehydrogenase. The inhibitor of lactic dehydrogenase appeared to be less stable than that of d-amino acid oxidase since the latter could be kept in the refrigerator for 7–10 days without appreciable loss in activity, whereas the inhibitor of lactic dehydrogenase frequently became inactive within 2–3 days.

DISCUSSION. The possibility that inhibitors such as those described for amino acid oxidase and lactic dehydrogenase may be factors in shock and anoxia has already been suggested (1, 2). They may also serve as regulators of metabolism under normal conditions in the intact tissue, or perhaps as protective mechanisms to decrease metabolism under conditions of O_2 lack. Under optimal conditions the rate of respiration of minced tissues appears to be of the order of the maximal rate as opposed to the resting rate occurring in the intact organ. Smythe (9) found that O_2 consumption of minced heart approaches the highest values for heart working at maximal effort. It seems possible that in the minced tissue the inhibitor may be diluted out and becomes less effective than in intact tissue, thus allowing maximal activity.

In a few experiments we tested the effect of extracts on carboxylase (yeast and animal). Some inhibition was produced in a few cases, but the inhibition did not parallel either that for amino acid oxidase or lactic dehydrogenase. It would seem possible that inhibitors are highly specific for the enzymes on which they act.

SUMMARY

A thermolabile globulin like substance which inhibits lactic dehydrogenase has been found in liver extracts. It appears to be similar to but not identical with the inhibitor of d-amino acid oxidase.

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STUDIES ON SHOCK INDUCED BY HEMORRHAGE

XIV. The Effects of Injection into Dogs of Amino Acid Oxidase Inhibitor[1]

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Blocking of metabolic processes is a well known occurrence in shock. One explanation for this is the breakdown of coenzymes which are necessary for normal metabolism (1, 2). Another possible mechanism by which metabolic reactions may be blocked has been found in *in vitro* investigations (3). We have found that liver extract contains a thermolabile globulin-like substance which inhibits amino acids oxidation *in vitro* by combining with the enzyme. Certain conditions which favour the inhibitor-enzyme combination *in vitro* have been investigated and appear to coincide with those occurring in shock (4).

The present investigation was undertaken to determine whether the injection into normal dogs of the partially purified liver extract which inhibited amino acid oxidation *in vitro* would lead to a blocking of amino acid oxidases and produce an increase in blood amino acids.

METHODS. Dogs were anesthetized with pentobarbital sodium (32 mg./kg.). Blood pressure was recorded from one femoral artery, blood samples for amino acid determinations were collected from the other. A branch of the portal vein was cannulated for the injection of the extract which was administered by gravity at a rate of 130–160 drops per minute.

The liver extracts were dialysed ammonium sulphate precipitates prepared as described previously (4). Control animals received either similarly prepared brain extracts which produced no inhibition when tested *in vitro*, or gelatin solutions. The gelatin solutions contained an amount of protein N comparable to that of the extracts and ammonium sulphate comparable to the small amount remaining in the tissue extracts after dialysis. The protein N content of the extracts varied between 0.6 and 1.1 mg./cc.

Blood amino acids were determined by the method of Krauel (5), modified slightly to allow readings to be made in the Cenco Photelometer. Protein and non-protein nitrogen in the extracts were determined by the micro-Kjeldahl method of Ma and Zuazaga (6) after metaphosphoric acid precipitation.

RESULTS. The results of the experiments are given in table 1. The blood amino acids increased in all of the five animals receiving liver extract. The smallest increase in these animals was 25%, the largest 222% and the average increase was 121%. Of the six control dogs which received gelatin or brain extract no change in blood amino acids was observed in 3 animals. One animal receiving gelatin showed an increase in blood amino acids of 28%, and one of the animals receiving brain extract showed an increase of 36% and one an increase of 28%. The increase in the last experiment (No. 63) was questionable

since only one sample in the middle of the series was above normal. The average increase in the control group was 15.4%.

TABLE 1
Liver extract

EXP.	DOG	EXT.	MG. PROTEIN N/KG.	ACTIVITY IN VITRO % INHIB.	TIME OF SAMPLING	AMINO ACID N MG./100 CC.	% INCREASE
	kg.				hours		
59	9.3	Liver	8.82	58.7	0	5.9	222
					1	5.9	
					2	8.0	
					3		
					4	8.8	
					5	8.5	
					6	10.5	
					7	10.0	
61	13.2	Liver	4.2	58.7	0	6.5	76.9
					0.5	6.75	
					2.5	8.2	
					3.5	9.05	
					4.5	8.0	
					6.0	11.5	
66	11	Liver	9.27	43.1	0	9.5	173.6
					1	10.25	
					2	11.25	
					3	12.9	
					4.5	13.0	
					6	26.0	
67	8.5	Liver	12.0	43.1	0	13.5	92.6
					1	16.5	
					2	18.0	
					3	26.0	
					4.5	21.0	
					6	Increased	
69	9.25	Liver	15.44	43.1	0	5.6	25
					1	5.6	
					2.5	5.9	
					4	6.4	
					5.5	7.0	
					6.0	7.0	

Average increase = 118%.

When liver extract was injected, it will be noticed that the rise in amino acids occurred almost immediately after the end of the injection and the increase continued until the end of the experiment. With brain extract or gelatin, in those experiments in which a small increase occurred, the change was not observed until several hours after the injection.

TABLE 2

Controls

EXP.	DOG	EXT.	MG. PROTEIN N/KG.	ACTIVITY IN VITRO % INHIB.	TIME OF SAMPLING	AMINO ACID N MG./100 CC.	% INCREASE
	kg.				*hours*		
58	11	Gelatin	7.64		0	7.5	0
					1	6.5	
					2	7.5	
					2.5	7.5	
					4.75	7.5	
					6.0	7.5	
					6.75	7.5	
60	19.2	Gelatin	8.76		0	8.2	28.1
					0.5	7.8	
					2.5	8.0	
					3.5	8.7	
					4.5	9.5	
					6	10.5	
62	7.55	Brain	11.4	6.5	0	6.25	36.0
					1	6.0	
					2	6.5	
					3	6.25	
					4.5	8.5	
					6	8.5	
					7	8.2	
63	8.4	Brain	10.25	6.25	0	9.75	28.2(?)
					1	9.75	
					2	10.5	
					3	12.5(?)	
					4.5	10.0	
					6	10.0	
					7	10.0	
64	9.8	Brain	13.6	0.0	0	7.5	0
					1	8.0	
					2.5	7.5	
					3.5	7.5	
					4.5	7.5	
					5.5	7.5	
65	9.3	Brain	13.6	0.0	0	9.0	0
					1	8.0	
					2 5	7.75	
					3.5	7.22	
					4.5	7.5	
					6.5	9.5	

Average increase = 15.4%.

The blood pressure occasionally fell during the periods of injection of the extract. However, they almost always returned to near the original level when the injection was stopped. In almost all instances the pressures at the end of the experiments were well above 100 mm. Hg. There was no apparent correlation between observed blood pressure changes and the blood amino acid levels.

DISCUSSION. It has been found in the series of dogs studied that the liver extract which inhibited the activity of *d*-amino acid oxidase *in vitro* leads to an increase in blood amino acids when injected into normal dogs. On the other hand, brain extract prepared similarly to the liver extract, but which had no effect on *d*-amino acid oxidase *in vitro*, and gelatin, when injected had little or no effect on the blood amino acids. It would seem probable that the increase in blood amino acids produced on injecting the liver extract is due to a blocking of the amino acid oxidases of the liver. Most of the *in vitro* work was carried out using the *d*-amino acid oxidase, because of its accessibility. However, it was found in experiments using whole tissue that the oxidation of *l*-glutamic acid was also inhibited by liver extract. It would, therefore, seem likely that both the *d*- and *l*-oxidases are blocked *in vivo*. The increases in blood amino acids following injection of the amino acid oxidase inhibitor are comparable to those observed by Engel *et al.* (7) in rats subjected to shock by hemorrhage.

The rate of injection and the concentration of the inhibitor were important factors, since dilute solutions or solutions injected slowly had little or no effect. Under these conditions the animal apparently was able to inactivate the inhibitor or to counteract its effect in some way.

In vitro experiments showed that the inhibitor formed an enzyme-inhibitor-substrate complex and that low pH and low phosphate concentrations favoured its combination with the enzyme, whereas high pH caused a dissociation from the enzyme, thus decreasing its activity. It was possible to reverse the effect of the inhibitor *in vitro* by change in pH. No attempt has been made to reverse its action *in vivo*.

An inhibitor of lactic dehydrogenase which appears to be similar to but not identical with that of *d*-amino acid oxidase has also been found in liver. It is possible that similar inhibitors for many enzymes may be naturally occurring. Since the inhibitors which we have studied appear to be normal constituents of liver they may function as regulators of metabolism under normal conditions. It is likely that in shock and anoxia conditions which favor their increased activity are produced, so that their effects are exaggerated and intermediary metabolites accumulate.

SUMMARY

The factor in liver extract which inhibited amino acid oxidase *in vitro* has been found to produce an accumulation of amino acids in blood when injected into dogs, presumably by a mechanism similar to that observed *in vitro*.

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THE EFFECT OF DIGITALIS UPON COAGULATION TIME OF THE BLOOD

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Recently Sokoloff, Ferber, and DeGraff (7) found that the average coagulation time in 10 cardiac patients did not decrease after digitalis therapy; their observations do not support the hypothesis that digitalis increases the coagulability of blood.

In the experiments to be described rather extensive investigation of the effect of digitalis on the coagulation time of the blood of normal animals yielded negative results except under the specialized condition of barbiturate anesthesia. An abstract of these studies appeared recently (8).

METHOD. In most of the work dogs of both sexes were used. In all procedures other than continuous injection with drug solutions, the dogs were kept on rigid feeding schedules and were neither bled nor dosed within several hours after feeding. In no cases were blood samples drawn at the site of drug injection.

Room temperature was kept constant during the course of each experiment; in most cases it was maintained between 28.0 and 29.0°C.

Coagulation time was measured directly by a modification of the Lee-White method. A 2-4 cc. sample of venous blood was drawn into a clean, oiled syringe. Samples obtained with even slight difficulty were discarded. Two 1 cc. portions of the blood were placed in dry 8 mm. tubes, of uniform diameter. The tubes were tilted at intervals until the blood no longer flowed; coagulation time was measured from the moment blood entered the syringe until the tube could be inverted. All measurements were made in duplicate. The technique was well standardized and the procedure carried out in all instances by the

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THE EFFECT OF DIGITALIS UPON COAGULATION TIME OF THE BLOOD

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Attention has been given in recent literature to the relation borne by digitalis and related substances to the clotting mechanism of the blood. In 1928 Tanaka (1) noted that strophanthin when injected intravenously into rabbits accelerated coagulation; it increased the thrombin content of the serum and the fibrinogen content of the plasma. Macht in 1943 (2) pointed out that heparinization of cats prior to injection of ouabain and of digitalis lowered the toxicity of these drugs. He also found coagulation time to be progressively shortened with an increased total of injected digitalis. Werch (3) reported a reduction of coagulation time in rabbits' blood by a single injection of Digifolin. DeTakats, Trump and Gilbert (4, 5) showed that digitalization of dogs markedly decreased the normal response to heparin. Cases have been presented by these authors in which embolic phenomena have appeared to coincide with therapeutic administration of digitalis to patients. It is suggested therefore that a disturbance of the normal clotting mechanism by the drug predisposes to the formation of thrombi. Massie, Stillman and Wright (6) reported that the administration of digitalis in therapeutic doses accelerated blood coagulation in each of 24 patients tested.

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same investigator. Over 3000 tests were performed in the course of the experiments here reported.

Prothrombin time was determined by Quick's method (9) modified in that each 0.1 cc. sample of plasma was diluted with 0.7 cc. isotonic solution of sodium chloride.

The tincture of digitalis used was in all cases freshly prepared from reference digitalis powder U.S.P. XII. This as well as the gitalin[1] used was diluted as indicated in the following section with Ringer's solution. In all experiments control solutions were prepared to contain alcohol in amount equal to that of the drug solution.

The following series of experiments were performed:

1. *In vivo:* (a) continuous intravenous administration of digitalis and of gitalin to dogs under Dial anesthesia, (b) continuous administration of digitalis to dogs under sodium pentobarbital anesthesia, (c) continuous administration of digitalis to dogs under ether anesthesia, (d) intravenous administration of a single large dose of digitalis to unanesthetized dogs, (e) oral administration of digitalis in amounts sufficient to produce mild intoxication, to unanesthetized dogs, and (f) digitalis assay on heparinized cats.

2. *In vitro:* (a) addition of digitalis to whole blood, (b) addition of varying amounts of heparin to blood containing digitalis, and (c) addition of varying amounts of digitalis to blood containing heparin.

RESULTS. *In Vivo:* (a) *Continuous administration of digitalis and of gitalin to dogs under Dial anesthesia.* Three sets of these experiments, each carried out on 7 experimental dogs, were performed; two sets of 7 control dogs were used. The animals were anesthetized with 0.65 cc. of Solution Dial-urethane per kg., injected intraperitoneally, and supplemented in amount as needed. The tincture of digitalis diluted 1:15 with Ringer's solution was injected intravenously at the rate of 1 cc. per kg. body weight every 5 minutes until the death of the animal. Coagulation time of blood drawn from the femoral vein was determined at 15 minute intervals for one hour before administration of the drug and throughout digitalization. Control animals were injected with appropriate amounts of alcohol in Ringer's solution in place of the digitalis preparation. Similar experiments were performed on 7 control and 7 experimental dogs using gitalin 1:5000 in Ringer's solution, prepared from a 1 per cent stock solution in 95 per cent alcohol.

During administration of digitalis the coagulation time of the blood was reduced from a value of 5.9 minutes immediately before beginning injection to a final value after 90 per cent of the fatal dose had been given, of 4.9 minutes, representing a 17 per cent reduction. These are average figures derived from the original data for all three experiments (21 cases). Statistical analysis of these data by determination of *t*-values (10) showed the difference between coagulation time before injection and that after the injection of 90 per cent of the fatal dose of digitalis to be significant; the decrease reached a statistically significant level (10.7 per cent) after a total injection amounting to 50 per cent of the fatal dose of digitalis. Control experiments showed no reduction in coagulation time. Similarly, during the injection of gitalin coagulation time dropped from 7.4 minutes before the drug was given to 5.6 minutes, a reduction of 24 per cent, although the calculated *t*-value was less than significant. Composite curves for these experiments are shown in fig. 1.

[1] Supplied through the courtesy of Rare Chemicals, Inc., Flemington, N. J.

In the third set of dogs given digitalis, in addition to determination of coagulation time, measurements of prothrombin time were also made.   In these 7 cases the decrease in coagulation time was less than that for the entire series of 21 cases; the difference between control and experimental curves was 0.2 minutes at the beginning of the experiment and 0.7 minutes after 90 per cent of the fatal dose had been injected.   Prothrombin time of the plasma was not altered by administration of digitalis (fig. 2).

(b) *Continuous administration of digitalis to dogs under sodium pentobarbital anesthesia.*   In fig. 3 are presented results obtained upon injection of digitalis in a manner identical to that used in the preceding experiment, into 7 dogs
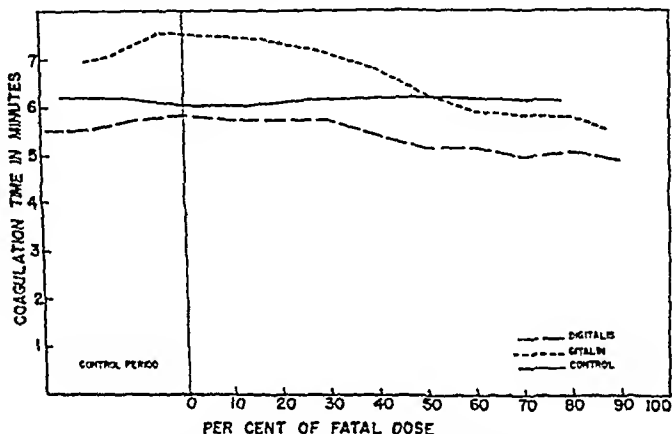
Fig. 1. Effect of Continuous Administration of Digitalis and of Gitalin upon Coagulation Time of the Blood of Dogs Anesthetized with Dial

Composite records for (a) 21 dogs injected with 1 cc./kg. tincture of digitalis diluted 1:15 every 5 minutes until death, (b) 7 dogs injected with 0.25 cc. gitalin 1:5000 every five minutes until death, and (c) 14 dogs injected with a control solution of alcohol every 5 minutes.

anesthetized with 30 mgm. sodium pentobarbital per kg. given intravenously, supplemented in amount as needed.   The coagulation time of the blood decreased in both control and experimental animals; however the fall after injection of digitalis was considerably greater than that for the control dogs.   The control curve dropped by an interval of 0.3 minutes while the experimental curve fell by 1.4 minutes, a difference of 1.1 minutes or 15 per cent.   Here again however, statistical proof of significance in the decrease of coagulation time occasioned by injection of digitalis under these circumstances is lacking, as judged by the *t*-value.

(c) *Continuous administration of digitalis to dogs under ether anesthesia.*   In these experiments the procedure was again repeated except for the anesthetic agent used, ether administered by tracheal intubation.   No marked alteration of the clotting power of the blood was apparent.   Control and experimental curves (fig. 4) did not differ materially from each other.

The difference in effect produced by digitalis on dogs anesthetized with ether from that on dogs anesthetized with Dial does not correlate with the fact that the fatal dose of digitalis is less under ether than under Dial. The average lethal dose of the tincture of digitalis given in this manner to etherized dogs
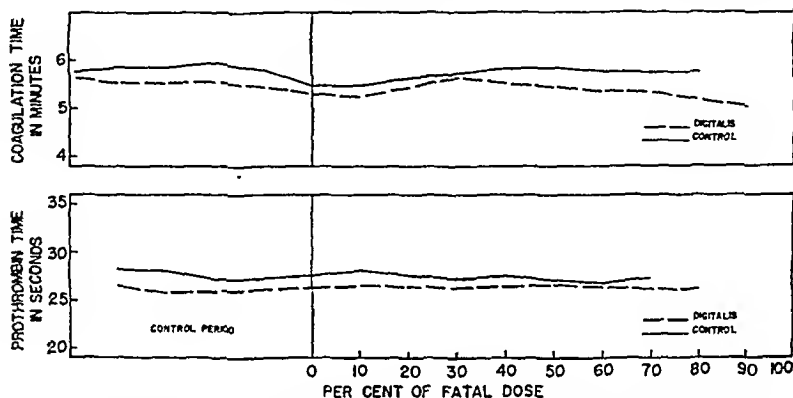
FIG. 2. EFFECT OF CONTINUOUS ADMINISTRATION OF TINCTURE OF DIGITALIS UPON COAGULATION TIME AND PROTHROMBIN TIME OF THE BLOOD OF DOGS ANESTHETIZED WITH DIAL

Composite records for 7 experimental and 7 control dogs
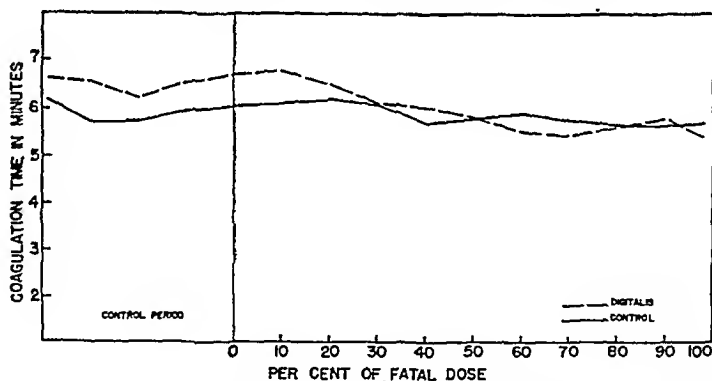
FIG. 3. EFFECT OF CONTINUOUS ADMINISTRATION OF TINCTURE OF DIGITALIS UPON COAGULATION TIME OF THE BLOOD OF DOGS ANESTHETIZED WITH SODIUM PENTOBARBITAL

Composite records for 7 experimental and 7 control dogs

was 0.94 cc. per kg., as contrasted with a lethal dose of 1.23 cc. per kg. for dogs anesthetized with the barbiturate. The lethal dose for dogs under Dial was thus 30.9 per cent greater than that for dogs under ether. The fatal dose for each individual animal is listed in table 1. These results resemble those obtained by Haskell (11) who found the fatal dose of Digifoline for cats, in two experiments, to be 30.4 per cent and 43.0 per cent greater respectively, under Dial-

urethane than under ether. Holck, Smith and Shuler (12) showed the mean fatal dose of digitalis (U.S.P. XI) for cats to be 51 per cent greater with Dial as the anesthetic, and 20 per cent greater with urethane as the anesthetic than it was with ether.

The average lethal dose of digitalis for the 7 dogs maintained under sodium pentobarbital anesthesia was 1.39 cc. per kg., 47.9 per cent greater than the average lethal dose with ether. Holck, Smith and Shuler found the lethal dose of digitalis for cats anesthetized with pentobarbital to be 47 and 49 per cent greater, in two experiments, than the lethal dose for cats anesthetized with ether.

(d) *Administration of a single large dose of digitalis to unanesthetized dogs.* Since the type of anesthetic agent used in the above experiments appeared to be a factor influencing the results it seemed advisable to investigate the effects of digitalis upon the clotting mechanism of unanesthetized animals. For these
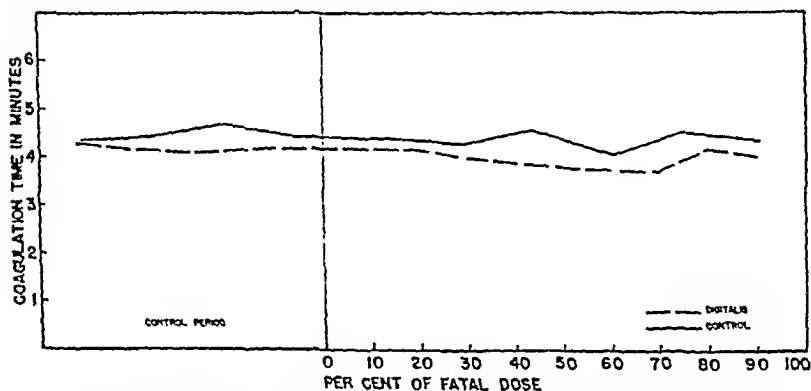
Fig. 4. Effect of Continuous Administration of Tincture of Digitalis upon Coagulation Time of the Blood of Dogs Anesthetized with Ether
Composite records for 7 experimental and 7 control dogs

experiments 7 dogs were trained to submit quietly to withdrawal of blood and injection of solutions. Coagulation time determinations were made at two hour intervals during 24 hours before administration of the drug and for 27 hours thereafter. Approximately one half the fatal dose was given, 0.5 cc. of tincture of digitalis per kg. body weight injected intravenously as a 1:5 dilution in Ringer's solution; this amount caused nausea and vomiting. Seven control dogs were injected with Ringer's solution containing alcohol in the same proportion as that present in the diluted tincture. When this experiment was carried out no reduction in coagulation time after injection of digitalis was seen; there was in fact a small increase in the average coagulation time during the period after administration of the drug (fig. 5). The difference between the two curves after 27 hours was 0.7 minutes, the t value for which indicated no significance.

In order to check the possibility that, in the previous experiment a transient effect of the drug had taken place and disappeared again during the two hour

TABLE 1

A comparison of the fatal dose of tincture of digitalis for dogs maintained under each of three anesthetic agents

ANESTHETIC	DIAL	SODIUM PENTOBARBITAL	ETHER
	Fatal dose in cc./kg.	*Fatal dose in cc./kg.*	*Fatal dose in cc./kg.*
	0.93	1.60	1.20
	1.55	1.60	0.87
	1.13	1.13	0.80
	0.73	1.40	1.07
	1.00	1.47	0.73
	1.20	1.20	1.13
	1.33	1.33	0.80
	1.80		
	1.20		
	1.33		
	1.20		
	1.13		
	1.40		
	1.27		
	1.20		
	1.07		
	1.07		
	1.87		
	0.93		
	1.40		
	1.20		
Average fatal dose..	1.23	1.39	0.94
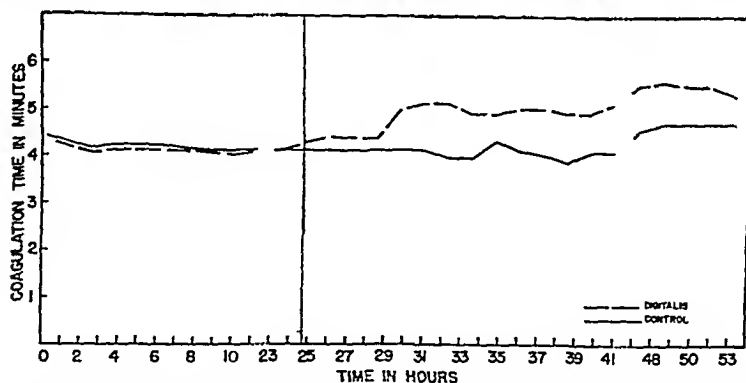
Fig. 5. Effect of a Single Large Intravenous Injection of Tincture of Digitalis (0.5 cc./kg.) upon Coagulation Time of the Blood of Unanesthetized Dogs
Composite records for 7 experimental and 7 control dogs

period between injection of the digitalis and the first coagulation test thereafter, the entire experiment was repeated with the exception that coagulation time determinations were begun immediately after injection of the drug and continued

at 15 minute intervals for two hours. Here again no effect was in evidence (fig. 6).

(e) *Oral administration to unanesthetized dogs of digitalis sufficient in amount to produce mild intoxication.* To approach more nearly the use of digitalis as it is administered clinically a series of dogs was given daily oral doses of digitalis. During a control period of 10 days coagulation time determinations were made at two day intervals on each of 7 dogs. Four experimental dogs were then put on a regime of daily oral dosage with 0.4 cc. of tincture of digitalis per kg. body weight given in gelatin capsules, this being continued until intoxication occurred, as judged by the appearance of nausea and vomiting. This required 5 days for most of the dogs. Two of the animals showed no signs of digitalis toxicity, and for these it was necessary to increase the dose to 0.6 cc. per kg. in order to produce symptoms of intoxication. After this point was reached
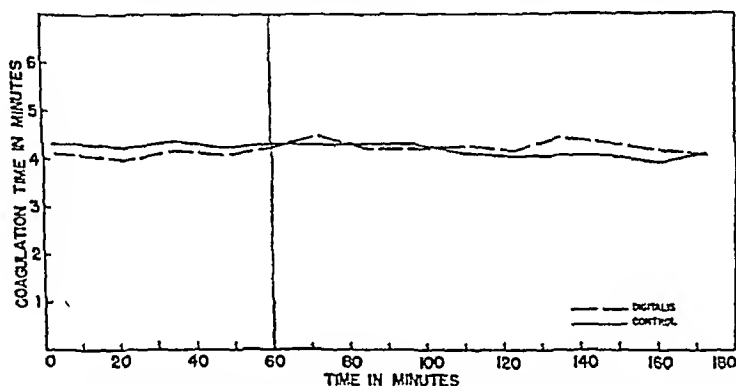
Fig. 6. IMMEDIATE EFFECT OF A SINGLE LARGE INTRAVENOUS INJECTION OF TINCTURE OF DIGITALIS (0.5 cc./kg.) UPON COAGULATION TIME OF THE BLOOD OF UNANESTHETIZED DOGS
Composite records for 7 experimental and 7 control dogs

the dose was reduced to one-half its former amount as a maintenance dose. Three dogs used as controls were given equivalent amounts of alcohol. Throughout the control period of 10 days, during the administration of the drug (15 days) and for 10 days after it had been discontinued, coagulation time records made at two day intervals on digitalized and control dogs remained comparable (fig. 7).

(f) *Digitalis assay on heparinized cats.* A series of 10 adult cats was used in an experiment similar to one reported by Macht (2) in which the effect of heparin on the toxicity of digitalis was investigated. The cats were injected intravenously with heparin sufficient in amount to render the blood incoagulable throughout the course of the experiment (1 cc. or 1000 units per kg.) and were then injected at 5 minute intervals with tincture of digitalis, diluted 7:150 with Ringer's solution, in the usual assay manner (U.S.P. XII) under light ether anesthesia. The lethal dose of digitalis for heparin treated cats was compared with that for a control series of 10 untreated cats. The lethal dose for

the group of heparinized cats was 0.72 cc. per kg. while that for the untreated cats was 0.79 cc. per kg. Thus the results of our experiment showed a tendency in the opposite direction from those obtained by Macht who reported an increase in the fatal dose upon digitalization. The t-value for the difference obtained in the present experiment between the mean fatal dose for heparinized and non-heparinized cats was 1.60 indicating a difference of no statistical significance.
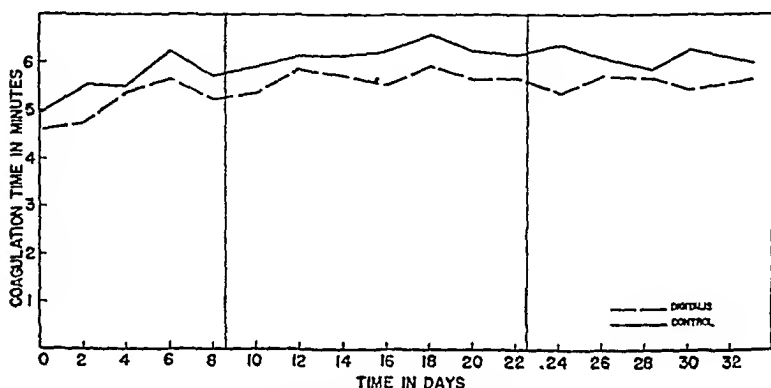
FIG. 7. EFFECT OF ORAL ADMINISTRATION OF TINCTURE OF DIGITALIS UPON COAGULATION TIME OF THE BLOOD OF UNANESTHETIZED DOGS

Composite records for 4 experimental and 3 control dogs   Vertical lines indicate beginning and end of drug administration

TABLE 2

Effect of addition of digitalis upon coagulation time of whole blood

NUMBER OF SAMPLES	ANESTHETIC AGENT	DILUTION OF DIGITALIS IN SAMPLE	AVERAGE COAGULATION TIME	
			Control	Digitalis
			min	*min*
10	none	1:1100	3 9	3.9
20	Dial	1:1100	4 1	3.9
20	none	1:110	3.9	3.9
20	Dial	1:110	3 7	3.9

In Vitro (a) *Addition of digitalis to whole blood.* When 0.1 cc. of diluted digitalis (either 1:10 or 1:100 in Ringer's) was added to 1 cc. of whole blood, and the coagulation time compared with that of control samples to which alcohol was added, the two figures showed practically no difference. This was the case for blood drawn from dogs under Dial anesthesia as well as for blood from unanesthetized dogs. The data for this experiment are listed in table 2.

(b) *Addition of varying amounts of heparin to blood containing digitalis.* To a series of tubes containing 0.1 cc. of diluted tincture of digitalis (1:10) or

control solutions of alcohol, was added 0.1 cc. of heparin in dilutions varying from 1:100 to 1:2400 of a stock solution in which 1 cc. equalled 1000 units. One cc. of freshly drawn blood from an unanesthetized dog was added to each tube and the coagulation time of the mixture determined. Coagulation time varied from 5 minutes to more than two hours according to the amount of heparin added, but there was no difference between the curves established for blood containing digitalis and blood containing a comparable amount of alcohol. No antagonism of digitalis to heparin could be demonstrated.

(c) *Addition of varying amounts of digitalis to blood containing heparin.* A similar experiment was set up in which the amount of heparin (0.1 cc. of heparin 1:2000) remained constant and the amount of digitalis varied. In this case 0.1 cc. of digitalis varying in strength from undiluted tincture to tincture diluted 1:100 was placed in the tubes, and 1 cc. of blood added as before. Here again the curve indicating coagulation time for blood containing digitalis duplicated that for coagulation time of the control blood.

This experiment was repeated with the single exception that a more concentrated solution of heparin (1:1000) was used. Once more there was no difference between the two curves.

DISCUSSION. In these experiments the clotting mechanism of the blood of normal dogs was not altered by the administration of digitalis unless given in amounts of 50 per cent of the fatal dose or more, and then only in certain instances and to a moderate degree, where a barbiturate anesthetic agent was employed. According to Ellis and Barlow (13) coagulation time undergoes a steady lowering over a period of many hours after the administration of a barbiturate anesthetic (barbital). In our experiments a slight fall was noted in the control series of dogs anesthetized with sodium pentobarbital which might be attributable to the influence of the anesthetic; a decrease in coagulation time beyond this extent in the experimental group of animals appeared to be due to the digitalis injected. In the control series of dogs anesthetized with Dial however no noticeable effect of the anesthetic on coagulation time occurred.

We have been able to confirm the finding of others that the use of barbiturate anesthetics lowers the toxicity of digitalis as judged by the increase in the fatal dose for animals under the influence of barbiturates as compared with etherized animals. One more bit of evidence of their influence upon the effects of digitalis is possibly to be seen in the fact just referred to that the coagulation time of the blood may be shortened by digitalis in animals anesthetized with a barbiturate, in contrast to negative results obtained in unanesthetized animals or those anesthetized with ether.

It may be pointed out here that although there is no apparent effect on the clotting mechanism as it operates under normal conditions there may yet remain an antagonism in vivo between the digitaloid drugs and any of several anticoagulant agents, principally heparin, added to the blood in the more complex methods of determining coagulation time. These factors may reasonably be excluded in the consideration of therapeutic administration of digitalis to patients.

SUMMARY

1. Continuous intravenous administration of a diluted tincture of digitalis (from U.S.P. XII reference powder) to dogs maintained under Dial anesthesia resulted in a reduction of blood coagulation time by 10.7 per cent after 50 per cent of the fatal dose had been injected, and a reduction of 17 per cent after 90 per cent of the fatal dose had been given; t-values for both of these figures were significant.

2. Continuous intravenous administration of gitalin to dogs under Dial anesthesia resulted in a 24 per cent decrease in coagulation time; the t-value for this decrease was less than significant.

3. Continuous intravenous administration of the diluted tincture of digitalis to dogs maintained under sodium pentobarbital anesthesia resulted in a 15 per cent drop in the coagulation time of the blood; the t-value was less than significant.

4. Continuous intravenous administration of the diluted tincture of digitalis to dogs maintained under ether anesthesia had practically no effect upon the coagulation time of the blood.

5. The fatal dose of the tincture of digitalis for dogs maintained under Dial anesthesia was found to be 1.23 cc. per kg., that for dogs under ether 0.94 cc. per kg., and that for dogs under sodium pentobarbital 1.39 cc. per kg.

6. Administration of a single large dose of the tincture of digitalis intravenously to unanesthetized dogs had no tendency to shorten the coagulation time and in fact tended to lengthen it somewhat several hours after injection of the drug.

7. Daily oral administration of the tincture of digitalis in amounts sufficient to produce mild intoxication to unanesthetized dogs had no effect upon the coagulation time of the blood.

8. Preadministration of heparin to cats did not alter the toxicity of the tincture of digitalis as determined by the usual assay method.

9. In vitro experiments showed no effect of digitalis on the coagulation of whole blood beyond that produced by a solution of equal alcohol content.

10. No antagonism between heparin and digitalis could be demonstrated in vitro.

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STUDIES ON ANTIMALARIAL DRUGS

The Metabolism of Quinine and Quinidine in Birds and Mammals[1]

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The metabolism of quinidine has received little attention compared with that of its optical isomer, quinine. In preceding papers of this series, we have shown that there is a quantitative difference in the ability of tissues of various species to metabolize quinine *in vitro* and that there is probably a relationship between the *in vitro* and the *in vivo* metabolism of the drug (1, 2). In the present paper we have extended this investigation to include *in vitro* and *in vivo* studies on the metabolism of quinidine. Furthermore, since there is some evidence to suggest that quinine is metabolized differently in the chicken and duck (3, 4), we have included both these species in our studies.

EXPERIMENTAL. *In vitro studies:* For the *in vitro* studies, the previously described procedure was followed (1): Briefly, tissues were blended in a Waring Blendor with 4 volumes of Ringer-Locke and filtered through gauze. Aliquots representing 0.2; 1.0 and 2.0 gms. of tissue were added to beakers containing 0.1 mgm. of quinine or quinidine in 10 cc. of Ringer-Locke. The total volume was adjusted to 20 cc. with Ringer Locke. The samples were incubated for 6 hours at 38–40°C., analyzed according to the procedure of Kelsey and Geiling (5) and the per cent destruction of the alkaloids determined. With the smaller species, pooled samples from several animals were used. With the larger species, at least two animals were used. Representative data are given in table 1.

In vivo studies: Adult rabbits, white leghorn chickens and white Pekin ducks were used. The animals were given 10 mgm./kg. intravenously of quinine or quinidine hydrochloride in 1% solution calculated as the free base. The drugs were injected into the marginal ear-veins of the rabbits and into the wing veins of the birds. The animals were sacrificed at intervals of 10 minutes, 1 hour and 4 hours after administration of the drug. The tissues were weighed and placed in 2% NaOH as rapidly as possible to minimize any *in vitro* destruction that might occur. The results are presented in Tables 2, 3 and 4.

RESULTS AND DISCUSSION. The *in vitro* experiments in the rat and rabbit show quinine to be more readily metabolized than quinidine. This is also apparent in the *in vivo* experiments in the rabbit. Ten minutes after the intravenous injection of the drugs, the concentrations of quinine observed in the various tissues, especially the liver, were significantly lower than with quinidine. The extremely low concentrations of quinine found in the liver may be a reflection of the ability of that organ to convert quinine *in vitro* with great rapidity. One and four hours after injection, the amount of quinidine remaining in the

tissues is still significantly higher than with quinine, although only small amounts of either are present at the end of four hours.

The *in vitro* experiments with bird tissues show quinidine to be more readily metabolized than quinine. The liver and kidney of the duck metabolized

TABLE 1

The quinine and quinidine oxidase activity in the tissues of various avian and mammalian species

The figures denote per cent destruction of 0.1 mgm. of quinine or quinidine after 6 hours incubation at 38–40°C. with 0.2, 1.0 and 2.0 gms. tissue.

TISSUE	SPECIES	QUININE			QUINIDINE		
		0.2	1.0	2.0	0.2	1.0	2.0
Liver	Chicken A	6	22	14	7	27	23
	Chicken B	0	8	6	0	8	10
	Turkey	10	25	15	15	75	80
	Pigeon	12	22	17	15	50	82
	Duck	14	50	30	7	50	84
	Goose	38	85	72	0	90	95
	Rat	87	97	98	0	12	16
	Rabbit	98	99	99	10	41	48
Kidney	Chicken A	0	20	21	12	77	93
	Chicken B	10	28	30	10	85	90
	Turkey	16	60	65	27	96	96
	Pigeon	9	27	34	0	50	96
	Duck	20	84	90	9	60	95
	Goose	44	95	94	10	96	96
	Rat		12	18	0	0	0
	Rabbit	25	75	85	0	0	0
Lung	Chicken A	0	0	0	0	7	8
	Chicken B	0	6	7	0	6	6
	Turkey	0	7	8	0	9	0
	Pigeon	6	15	15	0	0	0
	Duck	0	12	17	0	18	13
	Goose	0	8	12	0	8	0
	Rat		9	18	0	0	0
	Rabbit	28	93	98	0	10	10

Chicken A—results obtained Dec., 1944 from tissues pooled from 4 birds.
Chicken B—results obtained May, 1944 for tissues pooled from 4 birds.

both drugs with apparent ease while the results observed with chicken tissues showed them to be much less active in this respect.[3]

[1] Marshall (6) has demonstrated quinine oxidase in chicken liver in contrast to our earlier reports (1, 7). Our definite effects in at least one of the above experiments may be due to the fact that incubation was carried on at 40° instead of 37° as in our previous report or to physiological variations. Such variations have already been described in rabbits during pregnancy (2).

STUDIES ON ANTIMALARIAL DRUGS

The Metabolism of Quinine and Quinidine in Birds and Mammals[1]

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The metabolism of quinidine has received little attention compared with that of its optical isomer, quinine. In preceding papers of this series, we have shown that there is a quantitative difference in the ability of tissues of various species to metabolize quinine *in vitro* and that there is probably a relationship between the *in vitro* and the *in vivo* metabolism of the drug (1, 2). In the present paper we have extended this investigation to include *in vitro* and *in vivo* studies on the metabolism of quinidine. Furthermore, since there is some evidence to suggest that quinine is metabolized differently in the chicken and duck (3, 4), we have included both these species in our studies.

EXPERIMENTAL. *In vitro studies:* For the *in vitro* studies, the previously described procedure was followed (1): Briefly, tissues were blended in a Waring Blendor with 4 volumes of Ringer-Locke and filtered through gauze. Aliquots representing 0.2; 1.0 and 2.0 gms. of tissue were added to beakers containing 0.1 mgm. of quinine or quinidine in 10 cc. of Ringer-Locke. The total volume was adjusted to 20 cc. with Ringer Locke. The samples were incubated for 6 hours at 38–40°C., analyzed according to the procedure of Kelsey and Geiling (5) and the per cent destruction of the alkaloids determined. With the smaller species, pooled samples from several animals were used. With the larger species, at least two animals were used. Representative data are given in table 1.

In vivo studies: Adult rabbits, white leghorn chickens and white Pekin ducks were used. The animals were given 10 mgm./kg. intravenously of quinine or quinidine hydrochloride in 1% solution calculated as the free base. The drugs were injected into the marginal ear-veins of the rabbits and into the wing veins of the birds. The animals were sacrificed at intervals of 10 minutes, 1 hour and 4 hours after administration of the drug. The tissues were weighed and placed in 2% NaOH as rapidly as possible to minimize any *in vitro* destruction that might occur. The results are presented in Tables 2, 3 and 4.

RESULTS AND DISCUSSION. The *in vitro* experiments in the rat and rabbit show quinine to be more readily metabolized than quinidine. This is also apparent in the *in vivo* experiments in the rabbit. Ten minutes after the intravenous injection of the drugs, the concentrations of quinine observed in the various tissues, especially the liver, were significantly lower than with quinidine. The extremely low concentrations of quinine found in the liver may be a reflection of the ability of that organ to convert quinine *in vitro* with great rapidity. One and four hours after injection, the amount of quinidine remaining in the

chicken metabolizes quinine more readily than the duck. The *in vivo* experiments bear out this suggestion. At the end of four hours the concentrations of both quinine and quinidine remaining in the tissues of the duck are several times higher than those in the tissues of the chicken.

The discrepancy between the rates of *in vivo* and *in vitro* metabolism of the alkaloids in chickens and ducks led us to reexamine the specificity of our analytical method since one explanation of the results would be the presence of a substance in the duck tissues which would appear to be quinine (or quinidine) in the extracts.

Two control ducks and two ducks which had been injected four hours previously with 10 mgm /kg. of quinine were sacrificed and 20 gram aliquots of the

TABLE 4

The quinine and quinidine concentrations as mgm /kg. in various tissues of the duck at intervals of 10 minutes, 1 hour and 4 hours after the intravenous injection of 10 mgm /kg of the drugs

	10 MINUTES		ONE HOUR				FOUR HOURS	
	Quinine	Quinidine	Quinine		Quinidine		Quinine	Quinidine
Liver	34	60	42	46	28	37	24	16 5
Lung	12	23	15		13		6 2	6 2
Kidney	45	30	15		10	16	5 5	5.0
Spleen	50	60	22	18	18	17	6 0	5 5
Heart	19	16	7 1	7.1	5	5 8	2 0	2 0
Muscle	5 7	12	4 7		4		1.2	1 0
Brain	7.0	5 5	4 0		1 5	1 9	0 5	0 5
Bile	7 2	6 0	35		72		46 5	60
Pancreas	62	60	35		21	30	7 4	6 0
Bone marrow	9 8	7 2	5 0		2 5		1 0	1 4
Testis	16	14	19		15		16 0	9 0
Plasma	1 5	1 2	0 8	0 65	0 6	0 5	0 34	0 25
Red cells	5.8	4 3	2 1	2 0	1 9	1 0	0 7	0 5
White cells	12 0	14 2		10 4		12 0		2.0

liver and kidneys were extracted by our usual procedure. The N/10 H$_2$SO$_4$ extracts were made up to 25 cc. Absorption curves were obtained on these extracts and on a known solution of quinine in N/10 H$_2$SO$_4$ using a Beckman quartz spectrophotometer. Density values obtained with the extracts from the normal ducks were subtracted from those obtained with extracts from the treated birds before the specific absorption coefficients were calculated. The concentrations of quinine in the extracts were calculated from the D$_{2509}$ Å values. The specific absorption coefficients (α) are expressed as

$$\alpha = \frac{D}{c \times 1}$$

where

$$D = \log \frac{I_o}{I}$$
$$c = \text{concentration in gm./liter}$$
$$1 = \text{optical depth in cm.}$$

In an earlier paper (3), we reported that in the chicken, an intravenous dose of 10 mgm./kg. of quinine was almost completely removed from the tissues after four hours. Waletzky and Brown (4), however, reported that in the duck

TABLE 2

Quinine and quinidine concentrations expressed as mgm./kg. in various tissues of rabbits at intervals of 10 minutes, 1 hour and 4 hours after the intravenous injection of 10 mgm./kg. of the drugs

	10 MINUTES		ONE HOUR		FOUR HOURS	
	Quinine	Quinidine	Quinine av. 2	Quinidine av. 2	Quinine	Quinidine
Blood.......	2.9	3.7	0.3	1.5	0.06	0.14
Liver.......	1.3	16	1	20	0.13	1.2
Lung.......	88	140	54	89	0.6	3.0
Kidney... .	38	55	8	28	0.3	2.3
Spleen......	12	62	10	50	0.62	1.4
Heart.......	14	22	2	10	0.14	0.5
Muscle	7	10	2	6	0.15	0.2
Brain.......	2.9	2.8	1	1	0.28	0.1
Bile........			5	13	8.0	11.0

TABLE 3

The quinine and quinidine concentrations expressed as mgm./kg. in various tissues of the chicken at intervals of 10 minutes, 1 hour and 4 hours after the intravenous injection of 10 mgm./kg. of the drugs

	10 MINUTES				ONE HOUR			FOUR HOURS			
	Quinine		Quinidine		Quinine	Quinidine		Quinine		Quinidine	
Liver............	28	50	53	43	22*	22	42	2.6	2.2	2.6	6.5
Lung... ...	23		18		13	16		8.9		4.0	
Kidney	27		32		17	14		2.0		1.0	
Spleen..... ...	56	46	62	42	21	25	29	1.6	1.4	1.0	1.8
Heart...... ...	10	14	17	13	10	9	7	0.8	.44	0.3	0.7
Muscle........	17		7		5	3		0.4		0.08	
Brain....	11		9		3	1.7		0.2		0.14	
Bile......... . ..	3.5		33		29	96		72		126	
Pancreas....... .	52		55		21	27		2.1		2.3	
Bone marrow....	18		12		8	10		1.4		0.41	
Testis............	15		8		8	7		5.8		3.0	
Plasma..........	3.9	2.0		2.7	3.5		2.1		0.68		0.44
Red cells.........	5.4	3.7		4.0	2.0		1.8		0.2		0.2
White cells.......	37.5	24.0		24.0			8.0				7.2

* Av. of 5 birds (7)

"surprisingly high concentrations of quinine are present in the liver, the spleen and the brain, even 8 hours after a single intravenous dose of 60 mgm./kg.". Although Waletzky and Brown gave much higher doses than those used by us; and although neither method of analysis precludes the presence of some quinine degradation products, the data from the two laboratories suggest that the

the bird (chicken, turkey, pigeon, duck and goose), however, quinidine appears to be more readily metabolized of the two alkaloids.

In vivo, the rabbit metabolizes quinine much more rapidly than quinidine. In the duck and the chicken, the two drugs are metabolized at approximately the same rate. However, the duck metabolizes both alkaloids more slowly than does the chicken. A further difference in the two species is seen in the distribution of the drugs between red cells and plasma.

Further evidence of the specificity of the extraction procedure has been obtained.

Addendum. Recently, Hyatt & Quinn, J. Pharm. & Exper. Therap., 83, 101, 1945, reported that after intravenous injection, the four alkaloids, quinine, quinidine, cinchonine and cinchonidine were distributed in essentially the same fashion in the tissues of the dog.

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The data are given in figure 1. There is no indication of the presence of appreciable amounts of quinine derivatives in the extracts. Additional evidence of purity is supplied by the close agreement in the quinine content of the liver and kidney from the fluorescent analysis and from the spectrophotometric measurement at 2500 Å (liver, 18.5 vs. 17.9 mgm./kg.; kidney, 4.9 vs. 4.8 mgm./kg.).

A significant difference is apparent in the distribution of both drugs in the blood of the duck and the chicken when given under similar conditions. In
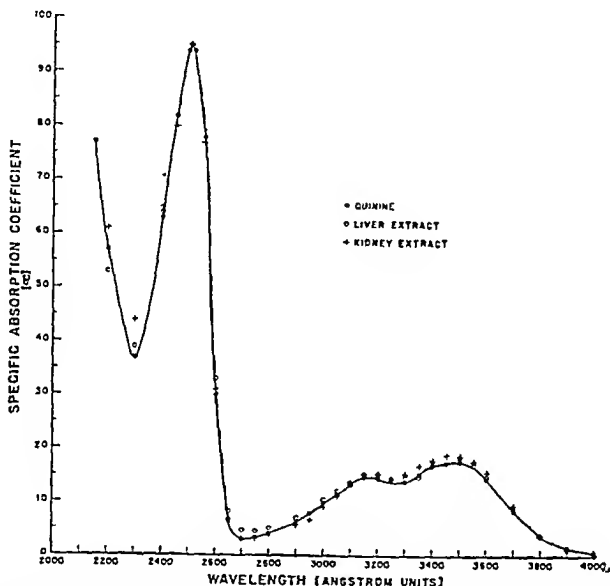
FIG. 1. ABSORPTION CURVES FOR DUCK LIVER AND KIDNEY EXTRACTS

Blank values obtained from extracted normal liver and kidney were subtracted from the density readings before the specific absorption coefficients were calculated. The concentrations of quinine in the extracts were calculated from the D_{2500} Å value. All readings were made in aqueous N_{10} H_2SO_4.

the chicken, one and four hours after administration, the concentration in the plasma is higher than in the red cells whereas the situation is reversed in the duck, the red cell concentration being appreciably higher than that of the plasma.

Comparisons between antimalarial activity of drugs in different species is complicated by differences in parasites and host-parasite relationships. In addition our *in vitro* and *in vivo* data suggest that the chicken and duck may handle drugs in an appreciably different fashion which might well affect the relative antimalarial activity in the two species.

CONCLUSION. Under the conditions of our *in vitro* studies, quinine is more readily metabolized than quinidine by the tissues of the rabbit and the rat. In

ration of NaOH was chosen after several trials to prevent undue colloidal aggrega-
the pentothal acid and still avoid making the solution too alkaline. With the high
ssociated with long injections, nevertheless, some necrosis of the tails of the mice
d. Different concentrations of the salt were injected into the tail vein at a con-
ate, utilizing a special injection machine (19) in which injections longer than one
were made possible by replacing the usual phonograph motor and turntable spindle
different spindle connected to a variable geared power supply.

the quickest injections used, the duration of which was estimated to be in the neigh-
d of one second, no machine was used. Instead, the plunger of the usual ½ cc.
was driven home by a taut rubber band fastened to the two arms of the syringe
and overlying the end of the plunger like a bowstring on an arrow. With this ar-
ient, the 27 gauge needle slowed the flow enough to cushion the movement, but the
is still so fast that the vein momentarily ballooned.

owing dilatation of the tail vein in warm water, each mouse received its dose in a volume
tional to its weight, equivalent to 0.005 cc. per gram of mouse. Calibrated syringes
ed for all injections. For the 30 sec. injection, the errors were within the following

TABLE 1
Ingredients in the pellet diet fed to "A" mice

nolasses	Ground yellow corn
oil meal	Ground hulled barley
an oil meal	Ground hulled oats
eal	Ground whole wheat
craps	Whole milk powder
ised buttermilk	Alfalfa leaf meal
luten feed	A and D feeding oil
l oats	2% salt
bran	½% steamed bone meal
flour middlings	1% precipitated chalk

e: The mix is sufficiently fine so that it appears the mice have no choice among the
ients.

THE EFFECT OF DIFFERENT INTRAVENOUS INJECTION RATE UPON THE AD50, LD50 AND ANESTHETIC DURATION OF PENTO THAL IN MICE, AND STRENGTH-DURATION CURVES O DEPRESSION[1]

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In the experimental evaluation of hypnotic drugs with reference to thei therapeutic safety, the first critical test is usually the determination of a rati relating lethal dose to effective dose, preferably $\frac{LD50}{AD50}$ (1–15). One or both o the doses involved in this ratio may be affected by a variety of conditions, such as number of animals used, strain, sex, nutrition, environmental temperature physical state of the drug, and rate of intravenous injection. (For references see de Beer et al. (16), Holck and Kanân (17)).

With respect to the factor of intravenous injection rate, it is generally recognized, both in the clinic and the laboratory, that the rate has an inverse effect upon the anesthetic and lethal dose, but quantitative data appear to be lacking in the case of the anesthetic dose and quite limited for the lethal dose. Swanson and Schonle (4) explored the variation in the lethal dose of amytal for rabbits and dogs over a considerable range of rates, and emphasize that a sufficiently rapid rate will almost halve the lethal dose in dogs. Das and Raventós (18), using evipal in mice, state that the median lethal dose depends upon the rate of intravenous injection. For a one minute injection they found the LD50 to lie between 150 and 180 mgm./kgm., but death could be produced by as little as 70 mgm./kgm. when this was rapidly injected. The present experiments were designed to study this problem in more detail by determining the effect of different constant rate intravenous injections upon both the AD50 and the LD50 of pentothal in mice.

METHODS. Previously unused male white mice, weighing 16–26 grams, were used in all of the experiments. The mice were obtained from two supply houses, each feeding a different diet. Except as otherwise noted, the particular diet upon which the mice were raised was maintained in the laboratory until the animals were used. The diet of the "A" mice used in the first series of experiments consisted exclusively of pellets which according to the manufacturer contain the ingredients shown in table 1. In the second series of experiments on the "B" mice, the diet was oats and hay supplemented with a commercial dog chow.

Care was taken to insure so far as possible standard conditions of hydration, hygiene, and environmental temperature. The pentothal was given as a freshly prepared solution of the sodium salt made up with an excess of NaOH, amounting to 27% more than that required to neutralize the pentothal acid, in order to minimize hydrolysis. This particular

TABLE 2

Median doses for different durations of injection of pentothal, together with calculated injection rates, $\frac{LD50}{AD50}$, and $\left[\frac{LD1}{AD99} - 1\right]\cdot 100$

DURATION OF INJECTION (min.)	AD50 "A" mice (mgm./kgm.)	LD50 "A" mice (mgm./kgm.)	LD50 "A" mice (mgm./kgm.)	LD50 "B" mice (mgm./kgm.)	RATE For AD50 "A" mice (mgm./kgm./min.)	RATE For LD50 "A" mice (mgm./kgm./min.)	RATE For LD50 "B" mice (mgm./kgm./min.)	LD50/AD50 Both doses given in a fixed time (Col. 1) "A" mice	LD50/AD50 Both doses given at the AD50 rate (Col. 6) "A" mice	SSM FOR FIXED INJECTION TIME (Col. 1) $\left[\frac{LD1}{AD99}-1\right]\cdot 100$ "A" mice (per cent)
1/60*	17.6 ±0.5	76.0 ±1.1			1056*	4560*		4.3 ±0.2		182
1/16	19.0 ±0.5				304					
1	19.0 ±0.6	82.9 ±1.1	Fed oats and alfalfa for 6 days prior to injections	82.6 ±1.7	152	663	660	4.4 ±0.2	5	220
½	19.1 ±0.5	98.1 ±1.9			76	392		5.1 ±0.3	6	230
3/8	20.3 ±1.0			117 ±4	53		293		10	
½		100 ±6.3				200		3.8 ±0.4	8	105
¾	23.8 ±1.2	112 ±8.8		139 ±7	32	149	185	4.7 ±0.6	9	90
2	28.6 ±1.7	171 ±2	207 ±11	243 ±6	14	86	122	6.0 ±0.4		230
5		221 ±50				44				
10	56.7 ±3.8	200 ±30		319 ±31	5.7	20	32	3.5 ±0.9		0

* Estimated value; injection time not less than ½ or greater than 1½ seconds.

The high standard errors for the 5 and 10 minute LD50 of the "A" mice reflect a smaller number of animals used, 16 and 17 respectively, and the shallow slope of the dosage-effect curves. Considerable care is required in these long injections to avoid secondary venepuncture during the struggling with resultant loss of some of the drug in the perivascular tissues. In the case of rapid injections, such extravasation is unlikely and easily detectable because the vein, which has been widely dilated by prior immersion in warm water, generally remains dilated during most of the injection, and the fast rate insures a visible swelling and whitening of the tail if leakage occurs. On the other hand, with longer injections and slower rates the tail tends naturally to blanche as it cools, the animal is more restless, and it is easy to lose the last fraction of the dose extra-venously without definite visible evidence. In later experiments on the "B" mice, the technique was improved by inserting the needle in the vein right up to the hilt, and exerting continuous pressure to maintain this relationship, which greatly decreased the labor of avoiding secondary venepuncture during struggling.

For the determination of any one median dose, experiments were conducted until two different doses with approximately even numbers of animals on each gave two percentage effects, one greater and the other less than 50%. Interpolation was then made by the method of Litchfield and Fertig (20). The number of animals involved in the calculations was 864.

RESULTS. The median doses of pentothal obtained with constant rate injections of different fixed durations are given in table 2. Both AD50 and LD50 are shown for the "A" mice, but for the "B" mice only LD50 were determined. Each median has been divided by the duration of injection to give the injection rate. For the "A" mice, two series of $\frac{LD50}{AD50}$ have been calculated; the first directly from doses experimentally obtained with the same duration of injection and therefore a proportionately faster rate for the LD50 as compared to the corresponding AD50; the second, by inspection of the graph in figure 1 in order to obtain an interpolated LD50 with the same rate of injection as each tabulated AD50.

In figure 1 the observed results with the "A" mice have been plotted in terms of the calculated rate against the duration of injection, which shows that for a given criterion of either anesthesia or death the two factors are roughly in inverse proportion to each other. Two lethal curves have been drawn in figure 1. That which adheres closely to all the experimental data provides the interpolated LD50 values (equal to rate × time) involved in the series of $\frac{LD50}{AD50}$ to be expected with the same injection rate for both AD50 and LD50 (table 2). The second curve fitted to the lethal data in figure 1 is the same as that fitted to the anesthetic data, and is given to show how both sets of data tend to follow the hyperbola, $y = Kx^{-0.8}$.

It may be seen in figure 1 that the ratio of distances from the y axis of any two points on a lethal and an anesthetic curve respectively, lying in a line perpendicular to the y axis, supplies an $\frac{LD50}{AD50}$ for some constant rate; and two similar points in a line perpendicular to the x axis give an $\frac{LD50}{AD50}$ for some constant duration of injection.

TABLE 2

Median doses for different durations of injection of pentothal, together with calculated injection rates, $\frac{LD50}{AD50}$, and $\left[\frac{LD1}{AD99} - 1\right]\cdot100$

DURATION OF INJECTION (min.)	AD50 "A" mice (mgm./kgm.)	LD50 "A" mice (mgm./kgm.)	LD50 "A" mice (mgm./kgm.)	LD50 "B" mice (mgm./kgm.)	RATE For AD50 "A" mice (mgm./kgm./min.)	RATE For LD50 "A" mice (mgm./kgm./min.)	RATE For LD50 "B" mice (mgm./kgm./min.)	$\frac{LD50}{AD50}$ Both doses given in a fixed time (Col. 1) "A" mice	$\frac{LD50}{AD50}$ Both doses given at the AD50 rate (Col. 0) "A" mice	SSM FOR FIXED INJECTION TIME (Col. 1) $\left[\frac{LD1}{AD99}-1\right]\cdot100$ "A" mice (per cent)
1/60*	17.6 ±0.5	70.0 ±1.1			1050*	4560*		4.3 ±0.2		182
1/16	19.0 ±0.5				304				5	
1/8	19.0 ±0.0	82.9 ±1.1	Fed oats and alfalfa for 6 days prior to injections	82.6 ±1.7	152	663	660	4.4 ±0.2	6	220
1/4	19.1 ±0.5	98.1 ±1.0			76	392		5.1 ±0.3	10	230
3/8				117 ±4			293			
1/2	26.3 ±1.0	100 ±6.3			53	200		3.8 ±0.4	8	105
3/4	23.8 ±1.2	112 ±8.8		139 ±7	32	149	185	4.7 ±0.6	9	90
2	28.6 ±1.7	171 ±2	207 ±11	243 ±0	14	86	122	6.0 ±0.4		230
5		221 ±50				44				
10	56.7 ±3.8	200 ±30		310 ±31	5.7	20	32	3.5 ±0.0		0

* Estimated value; injection time not less than ½ or greater than 1½ seconds.

Practically the same relation between rate of injection and time, for the lethal dose of amytal in rabbits and in dogs, may be calculated from the data of Swanson and Schonle (4). The weights of their animals are not given, but it is interesting to note that division of the calculated rates in mgm./min. by an assumed weight, 12 kgm. for dogs and 2 kgm. for rabbits, displaces the latter curves vertically to the region of the curves for mice (fig. 2). These rate-time curves for threshold responses to anesthetic drugs are, furthermore, similar to the classical strength-duration curves for threshold electrical excitation of the nerve or muscle cell, if the view is taken that intravenous injection rate (a function of drug concentration in the blood of the animal unit) is comparable
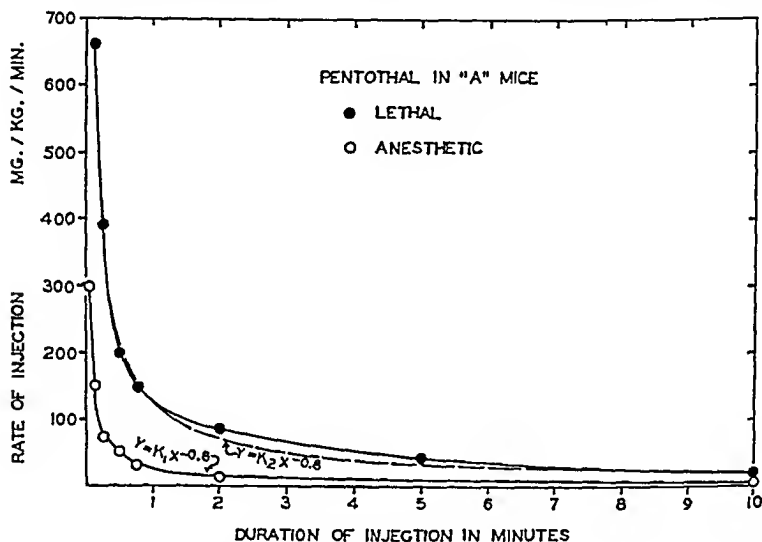
FIG. 1. RELATION BETWEEN RATE AND DURATION OF INJECTION REQUIRED TO PRODUCE A GIVEN DEGREE OF DEPRESSION

to electron flow rate or current (a function of electrical concentration in conduction systems of the cellular unit).

The similarity of the curves is best seen when the factors are plotted in logarithmic coordinates (fig. 2). The extent to which the plot is linear in these coordinates determines at a glance the suitability of the simple equation, $y = Kx^{-\alpha}$, in which K is a constant and α is equivalent to the slope of the line. The excitation data in figure 2 from Rushton (21) conform unusually well to this equation, with a value for α of 0.8. For excitation most values of α lie between 0.6 and 0.8, none being less than 0.5 or more than 1.0 (22).

It will be noted that the experimental data on the mice only tend to follow $y = Kx^{-\alpha}$, as in reality they oscillate around this relationship. In the case also of the electrical strength-duration curves, the equation is generally no more than a first approximation. It was at first thought that despite the reassurance
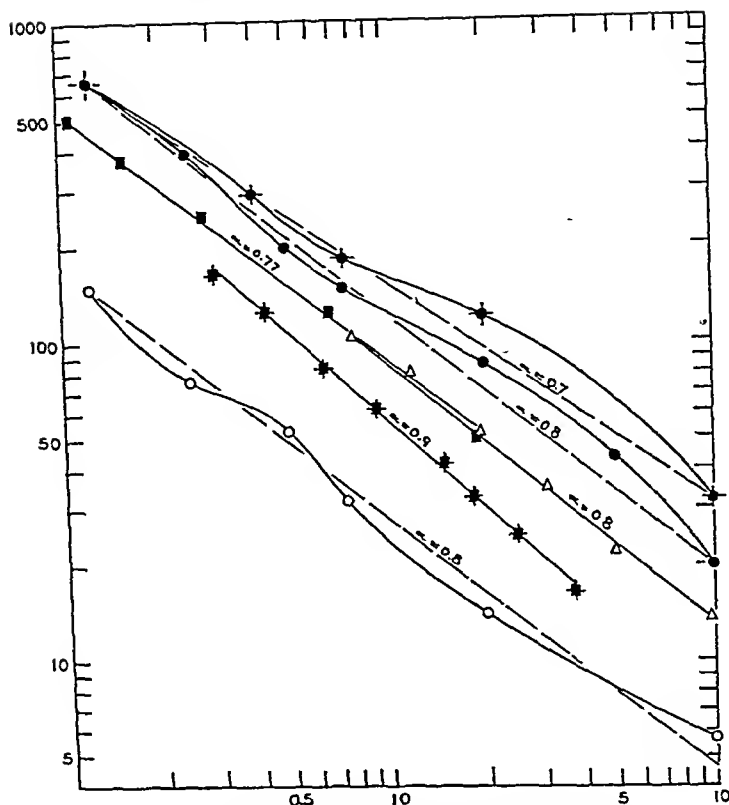
FIG. 2. SHOWING THE EXTENT TO WHICH THE DATA CONFORM TO $y = K x^{-a}$ LOGARITHMIC ORDINATES AND ABSCISSAE

SYMBOL	SUBJECT	EFFECT	ORDINATES	ABSCISSAE
✦	"B" mice	Death—20 minute criterion	mgm./kgm./min.	Minutes
●	"A" mice	Death—20 minute criterion	mgm./kgm./min.	Minutes
■	Rabbits	Death—not otherwise specified	mgm./kgm./min.	Minutes
✱	Dogs	Death—not otherwise specified	mgm./kgm./min.	Minutes
○	"A" mice	Anesthesia—15 second criterion	mgm./kgm./min.	Minutes
△	Frog sciatic	Excitation threshold	Volts	Seconds × 10⁻⁴

of the standard errors the aberration in the mouse data from $y = K x^{-a}$ might be due to some experimental error operative between different determinations of median doses, but not within a series of injections with which any one median

is determined, as for example the effect of diurnal variations in temperature possibly affecting hydration and mouse weight. The lethal doses were, therefore, repeated, although a failure in the usual animal supply necessitated the use of mice from another concern. These "B" mice, while showing a greater resistance to the anesthetic, confirmed the shape of the lethal curve to a remarkable degree (fig. 2).

The suspicion that diet was responsible for some or all of the difference between the "A" and "B" mice was tested by feeding the "A" mice for 6 days on the best quality of unrolled seed oats and cured alfalfa. Access to their usual pellet diet was provided, but this was largely neglected. Overnight the mice became much more lively, feces became harder and blacker, water consumption was markedly cut, and the urine decreased in amount and became much less rank. On the 6th day, the LD50 for the 2 minute injection was determined and
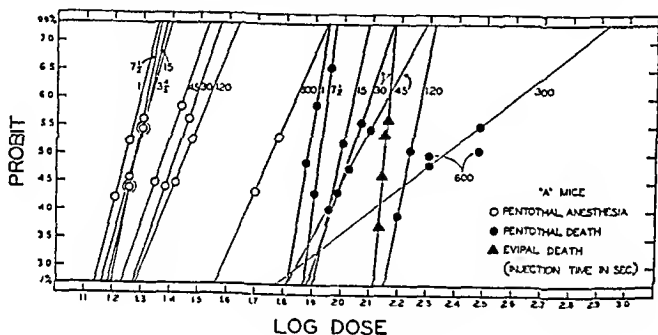
FIG. 3. RELATION BETWEEN SLOPE OF DOSAGE-EFFECT CURVE AND INJECTION TIME FOUND FOR "A" MICE

found to have been raised about $\frac{1}{2}$ of the 42% increase in dose which the "B" mice tolerated as compared to the "A" mice for that injection rate (table 2).

The dosage-effect curves for the different durations of injection of pentothal are given in figures 3 and 4. Those in figure 3 supply AD99 and LD1 for the "A" mice from which the "standard safety margin" (SSM) of Foster (9) can be calculated (table 2). No curve is given for the two dosages of the 10 minute LD50, since the range of percentage effect is too small for even a rough estimate. That this curve, nevertheless, has the most shallow slope of all is apparent from figure 3 and is further supported by an actual overlap of the lethal and minimal anesthetic doses. One of the mice receiving 60 mgm./kgm. for the determination of the 10 minute AD50 died 2 hours after the end of the injection. This death is considered particularly significant because all the mice receiving 60 and 75 mgm./kgm. for determination of the AD50 showed a toxic appearance characterized by pallor, convulsive movements, and severe respiratory depression; and deaths with doses used to determine the AD50 never occurred with injections given in 2 minutes or less.

The accuracy of the SSM values for the "A" mice is impaired by the com-

paratively few animals, usually about 30 and in two cases half this number, which make up each pentothal dosage-effect curve. Nevertheless, there are enough determinations to show that with fast injections the slope is steep and the SSM high in value, while with sufficiently slow injections the reverse effect is both indicated by the data for the 5 and 10 minute lethal injections (fig. 3) and confirmed by the demonstrable overlap of lethal and anesthetic dose found with the latter injection time. The more or less orderly transition from a high to a low slope is marred by the result for the 2 minute injection (fig. 3). The comparatively steep slope of the latter is associated with the highest $\frac{LD50}{AD50}$ for any injection time, and is probably not accidental since it was also found to occur with lethal doses in the "B" mice (fig. 4).

Also shown in figure 3 is the unusually steep curve for lethal doses of evipal determined with the same technique (injection time of 30 seconds), except that
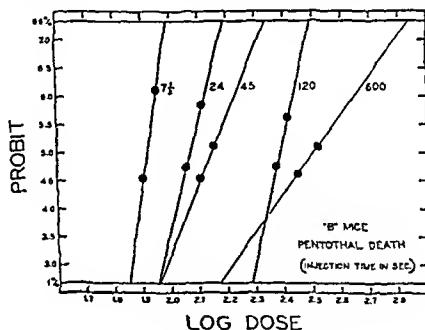
FIG. 4. RELATION BETWEEN SLOPE OF DOSAGE-EFFECT CURVE AND INJECTION TIME FOUND FOR "B" MICE

the sodium salt was prepared with only 15% excess NaOH and there were 89 animals approximately evenly distributed on 4 doses.

Some information on the relation between dose, duration of anesthesia, and injection time is contained in figure 5. Data are comparatively meager, partly because anesthetic durations were not consistently recorded, especially with long injections of pentothal which frequently lasted into the night, and also because not many doses were used in the center of the range between AD50 and LD50. Nevertheless, preliminary inspection of the data after plotting suggested that a given duration of anesthesia with pentothal required the same fraction of the difference between AD50 and LD50 for each injection time. The pentothal curves in figure 5 were drawn to meet this arbitrary specification and are presented, therefore, only to show that the results, while not conclusive proof, tend to support the hypothesis. Due to lack of space in the figure the numerous data on the individual durations of anesthesia in the region of the various AD50 values are not shown, but in the great majority of cases this was not over 3 minutes for injections not exceeding 45 seconds in duration.

With a fixed duration of injection, the injection rate is, of course, proportional to the dose, and the acceleration of anesthetic duration with increase in both dose and in rate is considerable. However, the independent influence of injection rate alone upon the duration of pentothal anesthesia, at least in the upper
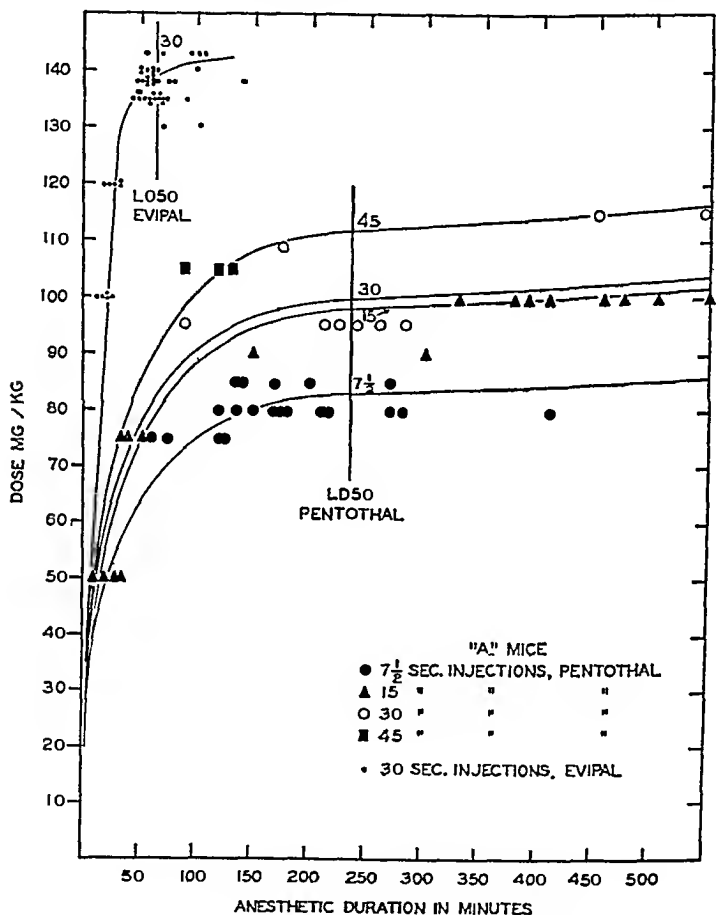
FIG. 5. RELATION BETWEEN DOSE, INJECTION TIME, AND DURATION OF ANESTHESIA

range of doses, is shown by the equally long anesthetic durations obtained with the increased injection rates incident to shorter injection times but with the same or smaller doses (fig. 5).

DISCUSSION. 1. *Relation between injection rate, therapeutic ratio, and duration of anesthesia.* The actual rates of injection in mgm./kgm./min. are frequently omitted in the literature on intravenous anesthetic and lethal doses of

barbiturates, and mention is seldom made as to whether a common injection rate or common injection time is used for the two doses entering into a therapeutic ratio. Since the injection rate affects both doses, the significance of differences in the ratio found for individual drugs under these conditions is questionable. In fact, the range of therapeutic indices of barbiturates reported in the literature was approximately covered in the present experiments using a single drug (pentothal) and different rates of injection for the AD50 and for the LD50.

For example, if all different combinations of pentothal AD50 and LD50 obtained for "A" mice with injection times of 45 seconds or less (table 2) are used to arrive at different $\frac{LD50}{AD50}$, the ratio may be anywhere from about 3 to 6. If the same injection rate is used for both doses, the ratio may be as high as 10, involving injection times of about 0.25 and 2.5 minutes for the AD50 and LD50 respectively. Furthermore, it was found that whether the AD50 and LD50 be determined with either the same injection rate or the same injection time, there was in the former case a particular rate, and in the latter a particular time, which gave a maximum $\frac{LD50}{AD50}$. Unless it should be shown that this rate, or this time, gives a maximum ratio for all hypnotic drugs which are rated by intravenous bioassay, one is obliged to conclude that two such drugs cannot be properly compared without rate-time curves (figs. 1 or 2) which detect maximum ratios.

Assuming that the most favorable $\frac{LD50}{AD50}$ is determined from appropriate anesthetic and lethal rate-time curves, such $\frac{LD50}{AD50}$ still may not supply an accurate idea of the margin of safety for the individual animal because it ignores the slopes of the dosage-effect curves. Foster (9) suggests as a standard margin of safety (SSM) the margin between the AD99 and the LD1. The results with pentothal, however, show that this margin fluctuates with the rate of injection, approaching zero with sufficiently slow rates (table 2). In the comparison of two different drugs, the SSM like the $\frac{LD50}{AD50}$ may thus depend upon different rates of absorption into the blood, whether directly by intravenous injection, or indirectly by absorption from subcutaneous sites or the gastro-intestinal lumen. Standardization of the absorption rate can be controlled in intravenous injections, and enough rates can be used to determine the most favorable $\frac{LD1}{AD99}$, or SSM, but the latter indices would still be of questionable value because they do not consider the duration of drug effect.

For example, Foster shows alurate by the intraperitoneal route to have an SSM in mice of 105% as compared to 34% for phenobarbital. It has been been found, however, that for the AD50 by this route phenobarbital produces an anesthesia in mice about 36% longer than alurate (23, 24), and this qualita-

tive difference is generally recognized (25). Consequently, for a given duration of action as long as that produced by the AD50 of phenobarbital, a smaller proportion of the margin between AD50 and LD50 is required with the latter drug; for to get this same duration of anesthesia with alurate would obviously require more than the AD50 of alurate. Similarly, for any given hypnotic fraction of the AD50 it seems reasonable to assume in the absence of contrary evidence that phenobarbital would have a longer action than alurate. The advantage inherent in the steeper slope of the dosage-effect curve of alurate may thus be cancelled out by its shorter duration of action.

These same considerations apply to a comparison of evipal and pentothal. From figure 5 it seems that for a given duration of anesthesia, especially if long, a smaller proportion of the LD50 is required in the case of pentothal than with evipal. But evipal appears to have a much steeper dosage-effect curve than pentothal (fig. 3), which in all probability (dependent on a correspondingly steep AD50 curve and the same $\frac{LD50}{AD50}$) means a higherS SM for evipal with this time of injection. For a given duration of anesthesia within the range possible with a 30 second injection of evipal, and on the basis of the fraction of the difference between the AD99 and LD1 required, it is thus problematical as to how great an advantage is possessed by pentothal.

In order to properly evaluate an intravenous anesthetic, or a hypnotic with no lag in onset of action (26) and which is to be tested by the intravenous route, it would seem advisable for preliminary bioassays to take into account the following interrelated factors: (1) The $\frac{LD50}{AD50}$ over a range of specified rates of injection in order to detect possible maximum ratios; (2) the shape of the dosage-effect curves involved in these ratios so that corresponding $\frac{LD1}{AD99}$ may be calculated; (3) the duration of anesthesia for a given fraction of the range between both the AD50 and LD50, and between the AD99 and LD1; (4) the quality of the anesthesia obtained.

2. *Relation between rate of injection, mode of detoxification, and slope of dosage-effect curve.* Brundage and Gruber (27) have shown in the dog that over 90% of the barbiturate (amytal, ortal, pentobarbital) injected intravenously leaves the blood stream in 1 minute, that within the next few hours the resultant blood concentration may be halved, but that these low blood concentrations, 0.0054% or less, are not related either to anesthesia or to death. They conclude that after intravenous injection the immediate fate of these barbiturates is distribution in the general body tissues, with subsequent release and assumed destruction in the liver.

Immediately after the ordinary brief intravenous injection, therefore, the nerve cells are largely protected from further barbiturate effect by distribution in the general body tissues which insures a low blood concentration of the drug. According to this view the lethal effect in mice of a very quick injection, e.g. 1 second, would depend essentially upon the detoxifying machinery (dilution, diffusion, fixation, etc.) available in the heart (via coronary circulation), lungs,

brain, and the portion of blood in the venous return which actually receives the drug from the needle. It may be mentioned that the manner of death in this case is exceedingly abrupt, quite as if the mouse were killed by a blow on the head.

On the other hand, with a sufficiently long injection involving a considerable increase in the recirculation of the drug, there is a much more even distribution of the dose throughout the body before death takes place. Accordingly, the maximal amount of animal structure participates in the same fatal end result. The low slope of the dosage-effect curve which accompanies the higher LD50 secured with a lower rate of injection may thus be explained as a broadening of the range of animal variation produced by the participation of more body mechanisms subject to potential variation. The definitely steeper dosage-effect curve found with the critically long 10 minute injection in the better appearing and more resistant "B" mice (fig. 4) is consistent with the assumption (28) that variation is less in healthier animals.

It is pertinent to recall in this connection that both the short and long injection have their counterpart in clinical anesthesia with the ultra-short acting barbiturates. As destinct from the usual comparatively quick injection, long operations with slow intravenous administration of evipal and pentothal are coming into favor (29–37). By analogy from the present experiments on mice, while the lethal dose for 50% survival may be trebled by a sufficiently slow injection rate, the hazard to the individual patient, as measured by the low slope of the dosage-effect curve, eventually becomes much greater than superficially appears.

3. *Similarity of the strength-duration curves of depression to those of excitation.* The significance of the classical strength-duration curves of excitation is that they represent a unique physiological evaluation of cellular state. This evaluation usually consists of an experimental determination of what quantitative combinations of electric potential and its time of application will just produce a given threshold cellular excitation. From the curve obtained, an index of its shape such as chronaxie may be derived. That electricity merely supplies a particular kind of energy to the tissue has been shown by Blair (38), who reproduced the strength-duration curve for threshold excitation of the frog's gastrocnemius nerve by substituting a jet of air pressure in place of the electric potential.

The virtue of utilizing two factors of energy, electrical or some other pressure and time, is that the evaluation thereby acquires a depth and perspective which may be likened to the advantages of binocular vision; it is a form of triangulation for the fixing of a point, which in this case is the evaluation of the excitation process itself in terms of a fixed reaction to different combinations of two energy factors. The importance of this method is emphasized by Frederique's statement (39) that a large number of physiological observations remained without correlation up until the general abandonment of DuBois-Reymond's dictum which limited analysis of cellular excitation to the single factor of electric current while ignoring the factor of time.

With respect to the further application of this general principle, it seemed not

unreasonable to suspect that when it is a question of altering the dynamic equilibrium of a protoplasmic unit in the direction of either excitation or depression, the factors governing the requisite exchange of energy might show a similar interrelationship, even for different vital units.

The justification, however, for comparing the barbiturate curves of depression with those of excitation rests on the following grounds: first, the subjects are comparable, since each represents a definite biological unit rounding out a cycle in ontogenetic development; in one case the nerve or muscle unit, in the other the whole animal as a unit. Secondly, the factors are comparable, for time is common to both, while electric potential involves a rate somewhat similar under the circumstances to the drug injection rate. This similarity resides in the fact that potential defines the rate of total electron movement, assuming other electrical conditions remain constant, and therefore measures the concentration of electrical energy capable of exciting a dynamic system. Likewise, the injection rate of the anesthetic determines the moment to moment blood concentration of what might be called drug energy, since the drug possesses energy capable of depressing a dynamic system. Finally, the criteria of the data are similar, for they are diametrically opposed threshold events, excitation and depression, which mark an all or none protoplasmic response to energy input.

The question arises as to whether the depression curves may be used in the same way for the animal unit as the excitation curves for the tissue unit, to provide a new and more sensitive evaluation of the physiological state of the animal as a whole in terms of the relationship between shifts in drug concentration and resultant shifts in the time required to produce a given threshold depression. An obvious handicap is that with the technique used in the present experiments, involving a population of animals rather than a population of measurements on the same animal, the analysis is limited to the detection of changes which may be induced in whole groups of animals for a time sufficiently long enough to perform the experiments. Nevertheless, the demonstrated effect of diet upon the lethal pentothal curve (fig. 2) gives some promise that the method may provide useful information. For example, the beneficial effects of the "B" diet apparently do not improve the operation of detoxifying mechanisms which function in less than a certain minimum length of time after the onset of drug injection, possibly the mouse circulation time; while on the other hand, the longer the injection time, the greater is the percentage by which the lethal dose for the "B" mice exceeds that for the "A" mice.

Of more immediate interest, perhaps, would be an improved correlation between type of drug, depth and duration of anesthesia, and ideal rate of intravenous administration, which might be obtained by analysis of anesthetic and lethal strength-duration curves (for most favorable ratios) taken in conjunction with accurate dosage-effect slopes and complete records of anesthetic duration. It is scarcely to be expected that the art of administration of an intravenous anesthetic would thereby be reduced to a calculated procedure, and doubtless this is indeed too much to expect so long as doses are introduced into a

dynamic system according to its weight alone, and without regard for the partition of this weight between important components such as blood volume, intercellular fluid, and visceral mass, or consideration for the factor of individual circulation time. Nevertheless, the prompt discard of calculated doses of intravenous anesthetics (40) is somewhat reminiscent of the confusion which surrounded the physiological evaluation of cellular excitability before the relation between current density and time was established.

With regard to the strength-duration curve of depression, it is desirable to have some explanation for its characteristic shape; in particular, from the standpoint of a strict inverse proportion, the depreciating value (higher figure necessary) of the time factor as compared to the strength factor when smaller strength values are used. Mathematically, this is reflected in the flattening of the curve (in ordinary coordinates) for longer times as the inverse proportion relation is progressively invalidated, and is also represented by the fractional value of α in the equation, $y = Kx^{-\alpha}$. In double logarithmic coordinates it is graphically represented by the extent to which the slope is less than $45°$.

Referring to the argument developed in the discussion of the distribution of barbiturate after intravenous injection, it was observed that with a maximally fast injection the structural dynamics of the animal are such that a minimum of the total animal structure is involved in the threshold depression. Conversely, with a sufficiently slow injection, maximum participation of all suitable animal structures takes place. If the additional structures called into action detoxify at a faster rate than that of simple diffusion, e.g. by chemical means in the liver, it is then to be expected that decreasing the injection rate would demand a continuously disproportionate increase in the figure for time, such as is signified by $y = Kx^{-0.8}$. The fact that the pentothal curves only tend to follow $y = Kx^{-\alpha}$, since in reality they oscillate around this relation (fig. 2) indicates that certain combinations of detoxifying mechanisms are somewhat more efficient than others when pitted against certain injection rates. By analogy from these considerations of the depression curve, the general shape of the excitation curve may also be due fundamentally to a change in the extent to which individual cellular structures with different rates of reactivity participate in the response at different rates of energy input.

<div style="text-align:center">SUMMARY</div>

1. The AD50 and LD50 of pentothal in mice were raised by decreasing the constant rate of intravenous injection.

2. The $\frac{LD50}{AD50}$ of pentothal varied from about 3 to 10, depending on the rates of injection used.

3. The $\left[\frac{LD1}{AD99} - 1\right] \cdot 100$ (SSM of Foster) was found to decrease for the same drug (pentothal) with sufficiently decreased injection rate.

4. Evidence is presented that a given duration of anesthesia with pentothal tends to require a fixed fraction of the margin (difference) between the AD50

and LD50 determined with a common injection time, despite variations in this time which alter both the AD50 and LD50.

5. In the upper range of doses with pentothal, increased injection rate without increase in dose prolonged the anesthesia.

6. As compared to mice raised on a commercially compounded pellet diet, those raised on a diet of rolled oats, hay, and dog chow supplement were found to be progressively more resistant to pentothal (LD50 increased by as much as 50%) as the injection rate is decreased. One-half of this difference was obliterated by feeding the former mice oats and alfalfa for 6 days.

7. It is suggested that preliminary bioassays of intravenous anesthetics, and of hypnotics with immediate onset of action which are to be tested by the intravenous route, include the following factors: (1) $\dfrac{LD50}{AD50}$ over a range of specified rates of injection in order to detect possible maximum ratios; (2) the shape of the dosage-effect curves so that corresponding $\dfrac{LD1}{AD99}$ may be calculated; (3) the duration of anesthesia for a given fraction of the range between AD50 and LD50, and between AD99 and LD1; (4) the quality of the anesthesia.

8. The factors of injection rate and injection duration were found to be related in a fairly regular manner for threshold anesthesia and death, approximately according to the expression, $y = Kx^{-\alpha}$, in which α varied from about 0.7 to 0.8.

9. The strength-duration curves of pentothal and amytal depression of the animal unit are similar to the strength-duration curves of excitation for the cellular unit.

10. It is suggested that the deviation in the shape of the depression strength-duration curves from the equilateral hyperbolic form may be accounted for by the more active participation in the response at slower injection rates of more remote body mechanisms which possess faster rates of detoxification.

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THE EFFECT OF ETHER, DIVINYL ETHER AND CYCLOPROPANE ANESTHESIA UPON THE HEART RATE AND RHYTHM AND BLOOD PRESSURE DURING NORMAL RESPIRATORY ACTIVITY AND DURING ARTIFICIAL RESPIRATION AFTER RESPIRATORY ARREST[1]

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In an earlier paper (1) it was reported that the cardiac irregularities which were observed in dogs at the time of, or soon after, respiratory arrest, produced by cyclopropane, were associated with anoxemia and that when artificial respiration was instituted the rhythm became normal and remained so even when the cyclopropane content of the blood was some 30% greater than that which produced respiratory arrest. However, if the concentration of cyclopropane was sufficiently increased cardiac irregularities returned even with an adequate arterial oxygen content present. Later Thienes *et al.* (2) reported studies showing that as one approached the stage of respiratory arrest irregularities developed and that by giving artificial respiration while increasing the content of cyclopropane in the respiratory system the arrhythmias were abolished. Other studies on animals have been reported in which cyclopropane was being administered by artificial respiration (3) but with the exception of the report by Schram (4) on ether we have found no such studies with ether and divinyl ether.

In an effort to compare the effects of cyclopropane, ether and divinyl ether upon the cardio-vascular system, as reflected by the EKG, and blood pressure we have repeated the procedure reported with cyclopropane, while using ether and divinyl ether.

EXPERIMENTAL PROCEDURE. Dogs (8-15 kg.) were used throughout this study. Preparations were made for recording heart rate, blood pressure, and respiratory rate and observing the EKG, lead 2, by use of a Sanborn cardioscope.

Anesthesia was induced by ether and divinyl ether by the open drop method, after which a tracheal catheter with cuff was inserted and from then on anesthesia was maintained by a closed system using a to-and-fro carbon dioxide absorber and a Foregger gas machine. In the experiments with cyclopropane a closed system was used *throughout*.

The anesthesia was gradually deepened over a period of an hour until Stage IV was reached. Blood pressure, heart rate, EKG and respiratory records were obtained at the various planes of Stage III and at Stage IV. Samples of arterial blood were taken from the dogs in the different levels of anesthesia and the concentration of the anesthetic agent determined by the iodine pentoxide method (5).

After respiratory arrest the dogs were placed in a respirator and artificial respiration started while increasing the concentration of the anesthetic in the system. At regular intervals thereafter records and blood samples were taken until the heart became irregular or stopped.
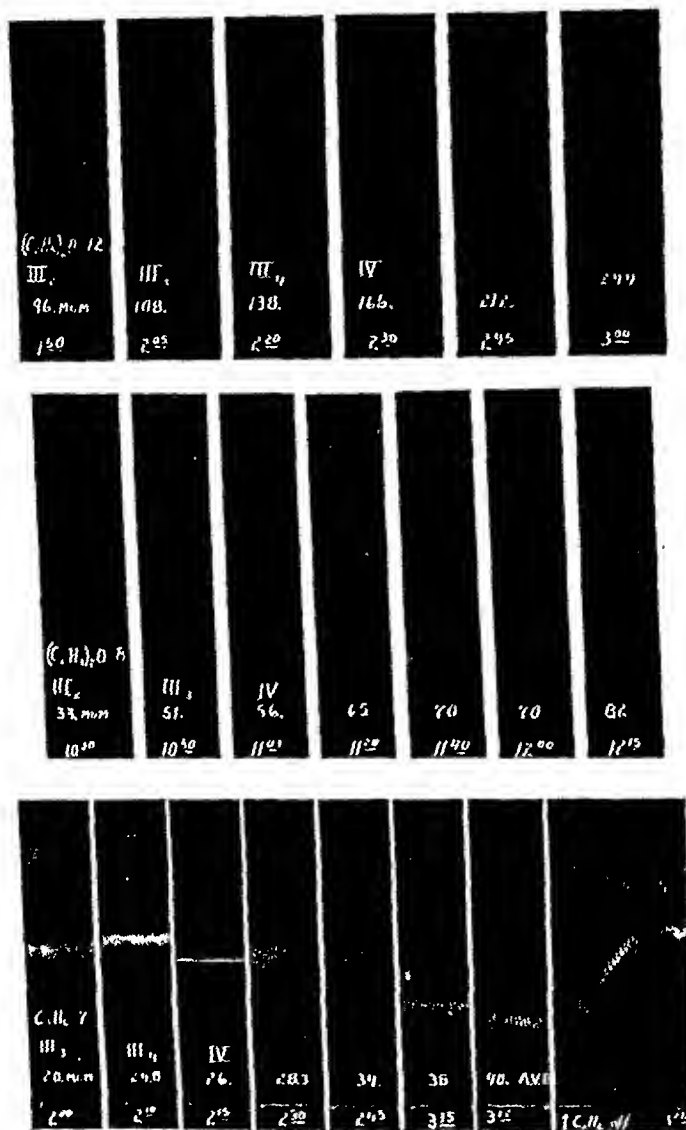
FIG 1. RECORDS OF EXPERIMENTS DESCRIBED IN TABLE 1

RESULTS. Protocols of a typical experiment of each of the three groups are shown in Table 1 with records of these three experiments shown in figure 1. In Table 2 the average results of the three groups of experiments are presented.

In Table 3 data are given regarding the oxidation reaction of ether and divinyl ether by iodine pentoxide.

TABLE 1

Protocols of individual experiments using ether, divinyl ether and cyclopropane as anesthetic agents

TIME	STAGE	ANES MGM 100 CC. BLOOD	Bp	HR	EKG	REMARKS
Diethyl ether—Dog 12—Weight 13.5 kg.—Anesthetic started at 1:20 p.m.						
1:50	III$_2$	96	150	180	Normal	
2:05	III$_3$	108	155	180	Normal	
2:20	III$_4$	138	135	180	Normal	
2:30	IV	166	95	190	Normal	
2:31	Respirator started					
2:45		212	50	180	Normal	
3:00		245	30	100	V.Ex.	
3:01	Ether off					
3:03					Normal	
3:10			80	210	Normal	
3:20	I		120	240	Normal	
Divnyl ether—Dog 8—Weight 14 3 kg.—Anesthetic started at 10:00 a.m.						
10:30	III$_2$	33	135	200	Normal	
10:50	III$_3$	51	130	160	Normal	Tremor
11:03	IV	56	100	200	A.V.Dis.	Tremor
11·04	Respirator started					
11:20		65	80	180	A V.Nodal	
11:40		70	55	210	A V.Nodal	
12:00		70	30	200	A V Nodal	
12.15		82	25	180	A V Nodal	
Cyclopropane—Dog 7—Weight 17 0 kg —Anesthetic started at 1·30 p.m.						
2:00	II1$_3$	20	150	105	S A.M.	
2:10	II1$_4$	24 8	160	120	S.A.S.	
2:15	IV	26	140	200	Normal	
2:17	Respirator started					
2:30		28 3	140	120	Normal	
2:45		34	140	110	Normal	
3:15		38	95	100	Normal	
3:22		40	80	80	A V.Block 1:6	
3:24	Cyclopropane off					
3:25 5					Normal	
3:26	Awake		170		Normal	

S A.M. = Sinus Arrhythmia Marked

S A S. = Sinus Arrhythmia Slight

Respiratory arrest was produced by ether with a concentration of 152 mg./100 cc. which is in the range of values given in the literature. Under artificial

TABLE 2

Table shows average concentrations of the anesthetics in mg./100 cc. arterial blood, blood pressure and EKG changes at different levels of anesthesia, and during artificial respiration

ANES.	NO. DOGS	STAGES OF ANESTHESIA													ARTIFICIAL RESPIRATION						
		III₁		III₂			III₃			IV			with EKG normal			with cardiac irreg.			av. final anes. conc.	
		mgm.	EKG	mgm.	Bp	EKG	mgm.	Bp	EKG	mgm.	Bp	EKG	mgm.	Bp	EKG	mgm.	Bp	EKG	mgm.	Bp
Ether	14	103	Reg.	120	125	Reg.	144	110	Reg.	152	80	Reg.	108	40	Reg.	232	30	Nodal V.Ex. V.Fib.	260	30
Divinyl Ether	14	33	Reg.	43	120	13 reg. 1 nod.	57	84	8 reg. 4 nod. 1 A.V. dis. 1 nod. tach.				72*	30*	Reg.*	68	35	Arrest A.V. black V.Ex.	82	20
Cyclopropane	8	16	Reg.	20	140	Reg.	25.4	145	Reg.	29.4	130	7 reg. 1 M.F. V.Ex.	40	100	Reg.	41		Nodal A.V. block V.Fib.		

* Only one dog.

Reg. = Normal rhythm
Nod. = A.V. nodal rhythm
A.V.dis. = Auriculo-ventricular dissociation
Nod.tach. = A.V. nodal tachycardia
M.F.V.Ex. = Multiple Focus Ventricular Extrasystole
V.Fib. = Ventricular Fibrillation

respiration a concentration of 198 mg./100 cc. was reached while the electro-cardiographic pattern was normal, although there was a marked lowering of the blood pressure. The rhythm of the heart became irregular with a concentration of 232, and the highest average concentration reached was 260 mg./100 cc. This value is in close agreement with that reported by Schram *et al.* for the cat under artificial respiration.

With divinyl ether cardiac abnormalities were observed in 6/14 dogs at the time of respiratory arrest. In none of these six dogs did the rhythm return to normal under artificial respiration while the anesthetic was being administered. In 7 of the 8 dogs with normal rhythm at the time of respiratory arrest the rhythm became irregular in 1–3 minutes after the respirator was started and

TABLE 3

Oxidation reactions of $(C_2H_5)_2O$ and $(C_2H_3)_2O$ by I_2O_5 and data showing percentage recovery

I. $5(C_2H_5)_2O + 12 I_2O_5 \rightarrow 20 CO_2 + 25 H_2O + 12 I_2$

II. $(C_2H_3)_2O + 2 I_2O_5 \rightarrow 4 CO_2 + 3 H_2O + 2 I_2$
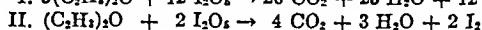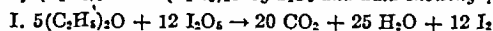
DIETHYL ETHER			DIVINYL ETHER		
Sample	Recovered	% recovery	Sample in air	Recovered	% recovery
In Blood					
10.8	10.2	94	11.4	11.1	97.5
10.8	10.2	94	5.3	4.8	90.6
10.8	10.2	94	19.2	18.3	95.5
5.4	5.3	98	6.7	6.8	101.5
5.4	4.9	91	10.7	10.4	97
5.4	5.1	94	10.7	10.8	101
5.4	5.1	94	4.4	4.5	102
4.3	4.2	98			
In air					
19.2	18.0	94			
25	23.7	95			
9	8.8	98			
14.2	13.9	98			
13.2	12.5	95			
Average.........		95.3			97.7

all 14 showed a rapid fall in blood pressure to about 30 mm. Hg. in 10–15 minutes, at which time the blood contained 68. mg./100 cc. The highest average concentration attained was 82. mg./100 cc.

There are no values given for Stage III, plane 4, for divinyl ether because in 12 of the 14 experiments generalized tremor or coordinated running movements obscured the signs for this plane. This muscular reaction has been investigated by Orth *et al.* (6) who have shown it to be present even after section of the cord at level of the 10th thoracic segment. These tremors are abolished at Stage IV and did not reoccur under artificial respiration.

Only eight experiments were done with cyclopropane because the results were similar to those of our earlier study (1). With this agent respiratory

arrest was produced by 29.4 mg./100 cc. blood and in 1 dog multiple focus ventricular extra systoles were present at the time of respiratory arrest.   This abnormal rhythm returned to normal upon starting the respirator.   Under artificial respiration it was possible to increase the cyclopropane some 33% above that necessary for respiratory arrest while the rhythm remained normal and while the blood pressure was about 100 mm. Hg.

Upon disconnecting the dog from the anesthetic system and continuing artificial respiration the return of the blood pressure and heart rhythm to normal and recovery of spontaneous respiration is most rapid after cyclopropane, intermediate with divinyl ether and slowest with diethyl ether.   This order is what one would expect in relation to the rates of elimination of the three agents as reported by Ruigh (7).

SUMMARY

The concentrations of ether, divinyl ether and cyclopropane necessary in the blood for various levels of anesthesia, and their effects upon the rhythm of the heart and blood pressure have been investigated.

The rhythm of the heart remained normal with ether or cyclopropane under artificial respiration, started at the time of respiratory arrest, until the concentration of these agents in the blood had reached a level of some 30–40% above that at the time of respiratory arrest, whereas with divinyl ether the rhythm was irregular in 6/14 dogs at respiratory arrest and became irregular in 7 more in 1–2 minutes after artificial respiration was started.

The blood pressure is maintained better under cyclopropane anesthesia than under diethyl or divinyl ether.

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STUDIES ON CYCLOPROPANE IX

The Effect of Premedication with Demerol upon the Heart Rate, Rhythm and Blood Pressure in Dogs under Cyclopropane Anesthesia[1]

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The use of Demerol as a substitute for opiates as preanesthetic medication has been proposed by Rovenstine and Batterman (1) who showed in experiments on dogs that it reduced the concentration of cyclopropane in the blood necessary for anesthesia and respiratory arrest, that it reduced the quantity of ether used to produce anesthesia and respiratory arrest, and that it prolonged the duration of anesthesia after a fixed dose of a barbiturate. They also reported its successful use in a series of patients.

Inasmuch as several reports (2) have mentioned its atropine-like and antispasmodic action, we were interested in observing its effect upon the rhythm of the heart when substituted for morphine and used with cyclopropane, because in previous reports (3) it has been shown that, in the dog at least, cardiac irregularities are routinely observed in cyclopropane anesthesia after premedication with morphine.

EXPERIMENTAL PROCEDURE. Dogs, 8–15 kg. weight, were used for this study. Preparations were made for recording blood pressure, respiration and observing electrocardiographic patterns (lead II) in the normal animal. Then 10 mg /kg. of Demerol was given intramuscularly and records made at 10–15 minute intervals for the next 30 minutes, after which anesthesia was produced by cyclopropane in oxygen and an intratracheal catheter with a Waters Guedel cuff inserted, following which anesthesia was continued by a closed system, using a Forreger gas machine with a to-and-fro carbon dioxide absorber. Gradually, during a period of 60–90 minutes the concentration of cyclopropane in the anesthetic bag was increased so that the dog was brought to respiratory arrest (IV). Samples of arterial blood were collected at the different planes of Stage III and at Stage IV for determination of the C_3H_6 content by the iodine pentoxide method (4). The images shown on the cardioscope (Sanborn) were under constant observation.

RESULTS. The results of a typical experiment are given in Table 1 and a summary of the 10 experiments is presented in table 2.

DISCUSSION. As a rule there was a moderate fall in blood pressure 10–20 minutes after the intramuscular injection of Demerol but the pressure returned to the control level in 30 minutes; and this level was maintained in Stage III$_{2-3}$, but fell in Stages III$_4$ and IV. Also, there was a decrease in heart rate during the 30 minutes after Demerol, which returned to or above the control level during anesthesia.

In contrast to the experiments where morphine was used as preanesthetic medication the rhythm of the heart, as observed by a cardioscope, remained

TABLE 1

Protocol of experiment no. 8, in which Demerol was given as a preanesthetic agent

TIME	STAGE	CYCLOPROPANE mg./100 cc. blood	BLOOD PRESSURE	HEART RATE	EKG
9:40	Control		120	92	Sinus arrhythmia
9:41	Demerol given				
9:45			100	90	Sinus arrhythmia
9:50			100	90	Sinus arrhythmia
9:55			100	80	Sinus arrhythmia
10:00			115	72	Sinus arrhythmia
10:05			115	70	Sinus arrhythmia
10:10			100	68	Sinus arrhythmia
10:12	Cyclopropane started				
10:15	Tracheal tube in place				
10:35	III₁		110	75	See text
10:45	III₂	10	120	100	Regular
11:00	III₃	18	115	100	Regular
11:15	III₄	22.4	100	100	Regular
11:35	IV	31.2	70	60	Regular

TABLE 2

Table shows the heart rate and blood pressure before and 10, 20 and 30 minutes after premedication with Demerol (10 mg./kg. IM), and during different levels of anesthesia produced by cyclopropane. Also the amounts of cyclopropane, mg./100 cc., in arterial blood necessary for the different levels of anesthesia are given.

EXP. NO.	CONTROL		10 MIN.		20 MIN.		30 MIN.		III₂			III₃			III₄			IV		
	HR	Bp	HR	Bp	HR	Bp	HR	Bp	HR	Bp	C₃H₆ mg.	HR	Bp	C₃H₆ mg.	HR	Bp	C₃H₆ mg.	HR	Bp	C₃H₆ mg.
4	150	130					100	125				88	100	16.5				80	95	25.8
5	70	135	88	120	80	135	80	135				95	125	15.6	95	110	24.6	100	90	29
6	88	170	100	160	100	155	100	150				104	140	17.8	160	120		180	120	30.5
7	85	120	100	80	60	120	70	120	86	120	11.2	84	110	12.3	104	100	24.6	180	120	29.2
8	92	120	90	100	72	115	68	100	100	120	10	100	115	18	100	100	22.4	60	70	31.2
9	112	110	80	155	84	150	88	150	100	140	9				120	130	24.6	180	110	32
10	100	145	120	95	96	95	120	95	90	100	9	120	85	18.6	100	80	26.4	72	80	31.8
11	120	160	76	145	68	140	64	135	80	125	10	100	100	16.5	68	95	24.2	60	95	29.7
12	104	130	88	140	80	140	74	145	80	150	9.1	120	135	13.7	120	100	25	120	90	31.8
13	88	130	84	110	72	120	64	120	64	130		62	125	18.7	100	125	22.2	120	110	29.2
Av...	101	135	92	123	79	130	83	127	85	126	9.7	98	115	16.4	107	106	24.3	115	96	30

normal after premedication with Demerol until after respiratory arrest in 8 of 10 dogs. In one dog, No. 8, the only abnormality was an A.V. block occurring

at each 60th to 75th cardiac cycle for about 5 minutes in Stage III, plane 2 The other irregularities were in dog No. 12 and these varied from a bigeminal rhythm with the second beat a ventricular extrasystole with a rate of 52 per minute to shifting pacemaker, A.V. dissociation and nodal rhythm with a heart rate of 110–120 during Stage III, plane 3 and 4 and at respiratory arrest. This abnormal rhythm was returned to normal in 2 minutes after Stage IV was reached, by giving artificial respiration while the dog was disconnected from the anesthetic mixture.

The amount of cyclopropane necessary for the different planes of anesthesia was reduced for Stage III_2 and III_3 but not for Stage III_4 or Stage IV when compared to the non-premedicated dog. These differ from those of Rovenstine *et al.*, who found that Demerol, 10 mg./kg., given intravenously 10 minutes before induction of anesthesia, reduced markedly the amount of cyclopropane necessary for Stage III_4 and Stage IV.

This difference is due to a difference in procedures in that Rovenstine and Batterman began their cyclopropane 10 minutes after an intravenous injection of Demerol and we started cyclopropane 30 minutes after an intramuscular injection of Demerol, and reached Stage III_4 and Stage IV some 90–120 minutes after the Demerol was given. In a separate experiment Demerol was given intravenously and the dog was observed for 75 minutes. During the first 50 minutes after injection he was markedly depressed, then he began to whine and struggle. Cyclopropane anesthesia was induced and the dog was brought to Stage IV over a period of 45 minutes, at which time the concentration of cyclopropane in the arterial blood was 33.6 mg./100 cc. In two other experiments cyclopropane anesthesia was induced 10 minutes after an intravenous injection of Demerol and the dogs were brought to Stage IV in 30 minutes at which time the concentration of cyclopropane in the blood was 24.5 mg./100 cc. These dogs were disconnected from the anesthetic mixture and permitted to breathe air for 20 minutes after which they were again brought to Stage IV with cyclopropane and oxygen over a period of 30 minutes, this time, some 100 minutes after the injection of Demerol, the cyclopropane necessary for respiratory arrest was 30.5 mg./100 cc. These observations, we believe, may explain the differences in our data and those of Rovenstine and Batterman.

SUMMARY

1. An investigation of the blood pressure and electrocardiographic changes has been made in dogs anesthetized with cyclopropane after medication with Demerol.

2. In most dogs there is a reduction in heart rate and temporary fall in blood pressure after the intramuscular injection of 10 mg/kg. of Demerol.

3. The cardiac irregularities routinely observed during cyclopropane anesthesia after morphine premedication in dogs are not observed after Demerol premedication.

4. Demerol reduces the amount of cyclopropane necessary for the lighter planes of anesthesia.

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THE DEGRADATION OF QUININE IN THE DUCK, CHICKEN AND DOG[1]

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A number of studies (1, 2, 3, 4, 5) employing different methods for the determination of quinine in blood, plasma and tissues of animals have been reported. These methods vary in the extent to which they exclude degradation products from the determinations. It has been possible in this study by using selected methods to obtain evidence that when quinine is administered to the duck, the chick, or the dog, at least two degradation products of quinine are present in the plasma and liver.

METHODS. Quinine was administered to the ducks and chicks either by the drug-diet method (6) for 3 days or by intravenous injection. There was no difference in the results obtained using the two routes of administration. Intravenous administration only was used in dogs.

Three chemical methods have been employed. The first, M.P.A., involves precipitation of proteins with metaphosphoric acid, centrifugation and estimation of total acid-soluble fluorescence in the supernatant fluid (7). The second, E.D.C., involves alkaline extraction with ethylene dichloride, addition of trichloroacetic acid and estimation of total alkaline-extractable fluorescence (8). The third, Dye, consists of alkaline extraction with ethylene dichloride, addition of methyl orange solution and colorimetric estimation of the concentration of substances which are sufficiently basic to combine with methylorange (9).

In many instances sodium chloride was added to the metaphosphoric acid supernate and uniformly resulted in nearly complete quenching of the fluorescence. This indicated that at most only a trace of the degradation product described by Kelsey et al. (10) was present. Alkaline wash of the ethylene dichloride and estimation of fluorescence in the ethylene dichloride before and after washing supports this observation.

All results are calculated as quinine base and expressed as micrograms per 100 cc. All determinations were made in duplicate.

Quinine added to plasma or liver from normal ducks or chicks could be recovered to the extent of 92 to 105 per cent by all three methods—M.P.A., E.D.C. and Dye. The same was found to be true for quinine added to dog plasma. The addition of quinine to the plasma of a duck which had received quinine resulted in satisfactory recovery of the added quinine by all three methods.

Quinine-oxidase was prepared in a crude form from rabbit liver by pressing out the liver in a Büchner press. This was diluted 3-fold with Ringer's solution. One cc. was used for each 10 cc. of plasma.

RESULTS. Table 1 summarizes the results of experiments with duck, chick and dog plasma. In duck experiments 1 to 5, ducks weighing about 200 grams were fed a diet containing 1/20 of a gram of quinine per 100 grams and were

bled on the third day after the start of the experiment.   In experiment 6, quinine was given intravenously.   In chick experiment 1, quinine was injected intravenously.   In the other experiments chicks three or five weeks old were fed a diet containing either 1/10 or 1/20 of a gram of quinine per 100 grams and the blood was obtained on the third day after the start of the experiment.   In the dog experiments various dosages were given and samples taken at various

TABLE 1

The concentration of "quinine" in the plasma of ducks, chicks and dogs

ANIMAL	METHOD	EXPERIMENT												
		1	2	3	4	5	6	9	8	9	10	11	12	13
		micrograms per 100 cc.												
Duck	M.P.A.	208	152	107	134	192	132							
	E.D.C.	92	69	60	39	110	44							
	Dye	28	30		22	87	26							
Chick	M.P.A	169	143	119	109	196	55	55	168	103	124	59	91	120
	E.D.C.	136	110	88	63	156	37	43	142	107	84	21	47	71
	Dye	159	117	91	70	166	36	40	65	50	120	64	80	100
Dog	M.P.A.	276	133	105	63	111	95	67	120	45	803	445	314	160
	E.D.C.	178	108	97	53	104	89	58	108	37	780	390	318	170
	Dye	188	95	96	54	89	84	69	121	43	750	354	298	96

TABLE 2

The concentration of "quinine" in the liver and plasma of ducks and chicks

ANIMAL	METHOD	EXPERIMENT							
		1		2		3		4	
		Liver	Plasma	Liver	Plasma	Liver	Plasma	Liver	Plasma
		micrograms per 100 cc.							
Duck	M.P.A.	17100	1100	24600	681	12090	310	9040	350
	E.D.C.	14400	740	21280	368	8512	156	6140	142
	Dye	9880	698	8530	258	6064	113	3140	111
Chick	M.P.A.	8120	458	1558	189	2980	240		
	E.D.C.	6040	284	860	167	1732	208		
	Dye	4171	271	408	140	1468	164		

intervals after injection.   No direct correlation between dosage or interval after injection was apparent: considerable variation in the ability of different dogs to degrade quinine appears to exist.

In table 2 are given the "quinine"[2] concentrations in the liver and plasma of

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[2] The term "quinine" has been used in referring to concentrations, calculated as quinine, given in the tables since several substances may be involved. Unless the degree of fluorescence and basicity of these substances is identical with that of quinine, the figures do not express absolute values of concentration.

ducks and chicks given quinine by intravenous injection. Blood and liver were obtained one and one-half to two hours after injection of quinine.

An oxidase is present in the liver of the rabbit and other animals (11) which converts quinine *in vitro* into an oxidation product which is probably a carbostyril (12). An attempt was made to use this enzyme as an analytical reagent. This was successful with duck plasma but not with chick or dog plasma. In the latter the complete decomposition of added quinine could not be obtained. Apparently the enzyme was quickly inactivated by some substance present in the chick and dog plasma. Four experiments were performed on the plasma of ducks injected with quinine. A typical experiment is summarized in table 3. With quinine added to normal duck plasma the E.D.C. and Dye method values show a 97 to 99 per cent loss after treatment with the liver. In the plasma of ducks injected with quinine about one-third of the fluorescence determined by the E.D.C. method remains after treatment with the oxidase while less than 10 per cent of the material determined by the Dye method remains.

TABLE 3

The concentration of "quinine" in duck plasma after incubation with quinine-oxidase

METHOD	A	B	C	D
	micrograms per 100 cc.			
M.P.A.	618	408	82	38
E.D.C.	285	103	6	6
Dye	216	21	19	7

A. Plasma of a duck injected with quinine, not treated with quinine-oxidase.

B. Same plasma as A treated with quinine-oxidase and incubated 5 hours at 37°C.

C. Normal duck plasma with addition of 600 micrograms per 100 cc. of quinine, treated with quinine-oxidase and incubated 5 hours at 37°C.

D. Same as C but with only 300 micrograms per 100 cc. of quinine added.

DISCUSSION. The data given in table 1 show that the fluorescence by the E.D.C. method is consistently less than that by the M.P.A. method in the plasma of ducks given quinine. This indicates that there is at least one acid-soluble fluorescent substance present which is not extracted from alkalinized plasma by ethylene dichloride. This substance resembles quitenine in its properties. Quitenine added to normal duck plasma behaves exactly like this substance.

The data in table 1 also show that in ducks given quinine the concentrations found with the Dye method are consistently lower than those found with the E.D.C. method. This may indicate that there are two substances present one of which is determined by the Dye method and both of which are determined by the E.D.C. method, or it may indicate that there is one substance present which is sufficiently basic to combine with methyl orange but more fluorescent than quinine. However, data typified by the protocol in table 3 show that over 90 per cent of the Dye-determined substances are lost after treatment with quinine-oxidase whereas approximately 67 per cent of the E.D.C.-determined

fluorescence is lost. This evidence strongly indicates that the presence of two or more substances is the explanation of the discrepancy between the E.D.C. and the Dye methods. Since the concentration of quinine in duck plasma can not be higher than the value given by the Dye method and since the quinine-oxidase has been found to decompose most of the dye determined substance there is some reason to believe that the Dye method gives a fairly accurate estimation of the concentration of quinine in duck plasma.

The behavior of quinine in the chick and dog appears to resemble qualitatively its behavior in the duck, but quantitative differences are present. In general, less of the two degradation products are present. With two substances, quinine and an unknown, extractable by ethylene dichloride, possessing different degrees of fluorescence, having different basicities, and present in varying quantities, it is not surprising that discordant results are found in chick and dog plasma. The results given in table 2 for "quinine" concentrations in the plasma and liver of ducks and chicks give further evidence that qualitatively the degradation of quinine in the two species is similar. Results given in table 1 indicate that the degradation of quinine in the dog may be qualitatively similar to that in the duck and chick.

CONCLUSIONS

1) There are probably at least two degradation products of quinine to be found in the duck after administration of quinine.

2) The degradation of quinine appears to be qualitatively similar in ducks, chicks and dogs.

3) Of the methods used the Dye method appears to give the most accurate estimate of the concentration of quinine in plasma.

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THE CHRONIC TOXICITY OF QUINACRINE (ATABRINE[1])

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Although the recent extensive use of quinacrine in the therapeutic and prophylactic treatment of malaria has focused attention on its possible chronic toxicity, no study of its lifetime effects on laboratory animals has been reported. Experiments (1, 2) conducted for shorter periods of time have shown that quinacrine can cause extensive pathological lesions in animal tissues, especially when relatively large amounts are administered. Early experiments (3, 4, 5, 6, 7) suggested and later work (8, 9, 10, 11) established the fact that an accumulation of quinacrine occurs in animal tissues. These observations indicate the possibility of damage from small dosages. Furthermore, if quinacrine is to be administered therapeutically or prophylactically over long periods of time, dietary considerations are likely to be important. Recently Scudi and Hamlin (12) reported that rats maintained on a high protein, low fat diet resisted the toxic action of quinacrine to a greater degree than those maintained on a low protein diet or a diet high in both protein and fat. Hegsted, McKibbin and Stare (13) found that the addition of various vitamins, yeast and protein did not prevent changes that were produced by quinacrine in animals on an already adequate diet. In the present investigation we have demonstrated differences in response to the toxic action of quinacrine when administered for the approximate lifetime of albino rats on high and low protein diets.

PART I. HIGH AND LOW PROTEIN DIETS. *Experimental.* Groups of 18 weanling rats each were selected at random with respect to litter mates from our colony of Osborne-Mendel strain. Six rats, 3 of each sex, were chosen from a litter and assigned to 10 different diets as required for balanced incomplete blocks of 3 with 10 treatments. The design was replicated as a whole 3 times, giving a total of 180 rats. Five groups were placed on a high protein diet and 5 groups were placed on a low protein diet. One group on each diet was given 100 ppm. of quinacrine; a second 200 ppm.; a third 400 ppm.; a fourth 800 ppm.; and a fifth, which was used as control, received the diet without the added quinacrine. The rats on the 100 and 200 ppm. were started at these levels. Those on the 400 ppm. were started at 200 ppm. and raised to 400 ppm. between 10 days and 2 weeks. Those on the 800 ppm. were started on 200 ppm. and in a like manner raised to 400 ppm. and then after another week raised to 800 ppm. The rats were kept in individual cages in a room with the temperature and humidity controlled for the duration of the experiment. The rations used had the following composition: The low protein diets contained dextrose 75%, casein 6%, corn oil 6%, brewer's yeast 5%, salt mixture (U. S. P. XII No. 2) 4%, whole liver powder 2%, and cod liver oil 2%. In the high protein diet the casein was increased to 30% with a corresponding reduction in dextrose.

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[1] Trade name quite commonly used.

The effect on growth. The first noticeable effect of quinacrine is an apparent dislike for the food containing it with a retardation of growth which may later be overcome by an adjustment to the distastefulness. Since many animals on the low protein diet with 800 ppm. of quinacrine died early, the interval of the first 12 weeks on the experimental diet was selected as the first period of study for a comparison of growth data from all groups. At this period the retarding effect

TABLE 1

Mean gain in weight of rats fed diets containing quinacrine

TIME IN MONTHS	DOSAGE OF QUINACRINE	SEX	LOW PROTEIN DIET		HIGH PROTEIN DIET	
			No. of Animals	Mean gain in weight	No of Animals	Mean gain in weight
	ppm			*grams*		*grams*
	0	M	8	177.4 ± 12.0	8	334.2 ± 13.6
		F	10	148.4 ± 12.1	9	217.0 ± 8.5
	100	M	7	162.7 ± 8.4	9	315.0 ± 10.5
		F	9	112.3 ± 6.1*	9	199.1 ± 5.7
3	200	M	8	157.1 ± 7.3*	9	289.4 ± 12.8*
		F	9	113.8 ± 6.7)*	9	192 1 ± 8.9*
	400	M	9	105.9 ± 7.0†	8	250.4 ± 13.7†
		F	9	101.2 ± 7.6†	9	159.5 ± 6.4†
	800	M	7	91.9 ± 6.4†	9	17.1 ± 9.7†
		F	9	73.7 ± 4.1†	9	132.8 ± 6.9†
	0	M	7	461.6 ± 26.9	6	488.3 ± 26.9
		F	10	288.8 ± 15.6	7	293.0 ± 13.8
	100	M	7	425.4 ± 13.5	8	495.2 ± 19.4
		F	8	232.9 ± 11.1†	8	294.6 ± 8.9
12	200	M	7	387.6 ± 15.0*	8	478.8 ± 20.4
		F	8	235.5 ± 9.5†	8	293.3 ± 10.5
	400	M	6	260.0 ± 4.1†	7	384.9 ± 21.5*
		F	4	208 3 ± 25.5†	6	222.7 ± 12.3†

* p < .05 — > .01
† p < .01.

of quinacrine on growth increases with the concentration of quinacrine (table 1). The early growth rate was retarded by all dosages used; however, the degree of retardation was not significant in all cases. At concentrations of 100 ppm. quinacrine retarded significantly the growth rate only of the female animals on the low protein diet. At the 200 ppm. level all values for retardation are significant except that for the male animals on the low protein diet.

In order to study the effect of quinacrine on growth for a longer period a second interval of the first year on the experimental diet was selected. By the end of the year all rats on the 800 ppm. of quinacrine had died. An analysis (table 1) for the groups on the low protein diet shows that there was a retardation at all concentrations; however, the value for the group of male animals on 100 ppm. of quinacrine remains nonsignificant. On the high protein diet quinacrine only at the dosage level of 400 ppm. caused significant retardation. The growth rate of the rats on the 200 ppm. of quinacrine had increased so that the value for the retardation of growth changed from significant at 3 months to nonsignificant at 12 months.

The effect on mortality. The mortality rate of the rats on the higher concentrations of quinacrine was related to the dosage of quinacrine and to the kind of diet (table 2). Quinacrine at 800 ppm. in the diet was very toxic. All animals on the low protein diet at this concentration died within 6 months. On the high protein diet the early mortality was less, but all rats died within a year. At the 400 ppm. level all rats in the low protein group died within 18 months and in the

TABLE 2

Per cent mortality of rats fed diets containing quinacrine

DOSAGE OF QUINACRINE	LOW PROTEIN DIET			HIGH PROTEIN DIET		
	6 mos.	12 mos.	18 mos.	6 mos.	12 mos.	18 mos.
ppm.						
0	5.5	5.5	33	11	27.5	44
100	11	17	39	5.5	11	39
200	5.5	17	61	0	11	55
400	17	39	100	11	27.5	83
800	100	100	100	11	100	100

high protein groups only 1 was living at the end of the experiment. In the groups on 200 ppm. of quinacrine fewer rats were living at the end of the experiment than in the control groups, but the difference is not significant (p. = 0.15).

Hematology. At intervals of 6 weeks blood studies were made on 6 rats from each group until too few survivors remained in a group to continue the studies. Table 3 gives the observations at 6 month intervals. The outstanding change was a leukocytosis, predominantly neutrophilic. It was marked in the groups on 800 ppm. of quinacrine, less striking in the groups on 400 ppm. and scarcely noticeable in those on 200 ppm. A slight increase in hemoglobin concentration and erythrocyte counts was noted in the groups on 800 ppm. of quinacrine.

Confirming the observations of Seigel and Mushett (1) the lymphocytes showed rounded basophilic granules within the cytoplasm (figure 1). In the earlier weeks of the experiment as many as one-half of the lymphocytes in the 800 ppm. groups contained these granules. Smaller percentages were seen in the groups on the lower dosages of quinacrine. In later observations the percentages gradually decreased until by the end of the first year the granules were rarely seen.

Pathology. Microscopic examination was made of 114 of the 180 rats. Lung, heart, liver, spleen, pancreas, stomach, small intestine, colon, kidney, adrenal and testis were sectioned routinely; thyroid, parathyroid, leg muscles, bone and marrow of tibia and femur, lymph node, brain, salivary glands, uterus and ovary

TABLE 3

Mean hematological effects of quinacrine at intervals of 6 months

WEEKS ON EXPT.	QUINA-CRINE IN DIET	NO. OF ANIMALS	HEMOGLOBIN (KLETT METHOD)	ERYTHROCYTES	LEUKOCYTES	NEUTROPHILS
High protein diet						
	ppm.		*grams*	*millions per cu. mm.*	*thousands per cu. mm.*	*per cent*
25	None	6	$17.1 \pm .48^*$	$7.8 \pm .25$	13.6 ± 2.2	23.3 ± 4.5
50		6	$18.4 \pm .57$	$8.4 \pm .57$	12.8 ± 1.2	27.6 ± 3.3
76		6	$16.8 \pm .30$	$9.0 \pm .70$	10.6 ± 2.2	38.3 ± 6.4
25	200	6	$17.7 \pm .53$	$8.6 \pm .17$	14.2 ± 1.4	28.3 ± 5.2
50		6	$18.7 \pm .14$	$8.9 \pm .39$	14.6 ± 1.4	29.5 ± 1.1
76		5	$16.6 \pm .61$	$9.5 \pm .42$	14.2 ± 3.4	39.8 ± 2.0
25	400	6	$18.7 \pm .34$	$8.7 \pm .13$	15.6 ± 2.0	28.3 ± 2.3
50		6	$16.4 \pm .53$	$7.6 \pm .26$	26.6 ± 3.1	42.1 ± 5.6
76		3	$15.6 \pm .47$	$7.5 \pm .37$	14.4 ± 2.3	35.6 ± 1.5
25	800	6	$17.8 \pm .53$	$8.8 \pm .24$	28.4 ± 5.7	48.5 ± 8.9
44		3	21.0 ± 1.90	10.2 ± 1.40	21.7 ± 9.9	47.3 ± 4.1
75		No survivors				
Low protein diet						
25	None	6	$17.1 \pm .34$	$8.6 \pm .28$	10.9 ± 1.0	30.0 ± 4.7
50		6	$18.9 \pm .42$	$8.4 \pm .18$	17.6 ± 1.8	29.3 ± 3.9
76		6	$16.3 \pm .53$	9.7 ± 1.00	14.6 ± 3.4	34.8 ± 7.2
25	200	6	$16.7 \pm .26$	$7.9 \pm .14$	11.2 ± 0.5	25.5 ± 2.0
50		6	$18.2 \pm .27$	$8.4 \pm .23$	14.5 ± 2.5	34.0 ± 3.6
76		6	$14.5 \pm .30$	$7.2 \pm .35$	11.3 ± 1.5	43.1 ± 4.2
25	400	6	$16.2 \pm .56$	$8.2 \pm .35$	21.7 ± 1.6	32.6 ± 3.5
50		6	$17.0 \pm .44$	$7.9 \pm .21$	22.9 ± 1.9	40.6 ± 2.7
76		No survivors				
13	800	5	$11.1 \pm .70$	7.4 ± 1.52	69.9 ± 7.1	67.6 ± 8.2
25		No survivors				

* The number following each mean is the standard error.

were sectioned less frequently. In addition to the usual hematoxylin-eosin stains on paraffin sections of formalin-fixed material, Perls' acid-ferrocyanide reaction with a basic fuchsin counterstain was used to indicate the nature of various pigments, and a Sudan IV stain on frozen sections was used for demon-

strating fat. A small amount of work with other fixatives and stains was done for the purpose of noting the reactions of the peculiar basophilic granules to be mentioned later.

Brief notes on the lesions seen in our rats following the feeding of high levels of quinacrine have been published (14, 15). Since many of the details of similar lesions have been described in 2 papers dealing primarily with the structural changes, namely, those of Wright and Lillie (2) and of Seigel and Mushett (1), many changes in our rats will be only briefly described, with more attention to those points not mentioned in the previous publications. Seigel and Mushett (1) found "slight adrenal and myocardial alterations" in rats given 1% of the LD50 for 15 months. They also noted moderate myofibrosis and basophilic granulation in various locations in rats given 2% of the LD50 for 4 to 6 months. The next higher dose, 5% of the LD50, killed the animals after 6 to 12 weeks, while still higher doses caused death after various shorter periods. Hegsted,
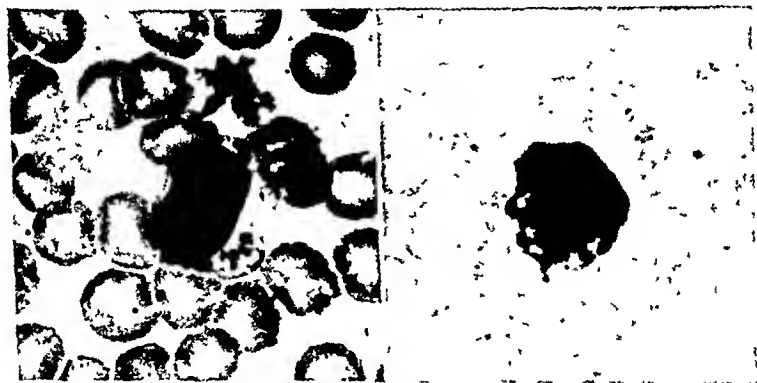
Fig 1 Basophilic Granules in Lymphocytes (× 1700, Wright's Stain)

McKibbin and Stare (13) examined microscopically "a few rats" which had received quinacrine at levels of 25 and 40 mgm /100 grams of diet for 6 months and found only minimal changes, essentially limited to basophilic granulation; no necrotic changes were detected Barlow, Auerbach and Rivenburg (9) briefly discuss the pathology in connection with pharmacological studies in rats which received quinacrine for 70 days They found that 5% of the LD60 given daily by stomach tube for 49 days caused insignificant findings at necropsy. Wright and Lillie (2) continued their experiments for 50 days All of the above named workers except Hegsted et al used the weight of the animal in computing the dosage level while our levels were computed as percentages of the diet, making a direct comparison of mortalities at a given dosage level somewhat difficult. The 40 mgm /100 grams of diet used by Hegsted et al corresponds to our 400 ppm.

Among our rats fed 800 ppm quinacrine in the diet, those on the low protein diet were severely affected much sooner than those on the high protein diet. The

first death in the low protein group occurred after 6 weeks with changes essenti-
ally those of inanition. All but 1 of the remaining 17 rats died or were sacrificed
because of poor condition after 13 to 19 weeks, while the last rat lived 21 weeks.
In addition to a generalized yellow discoloration, all 17 rats showed at autopsy
large irregular areas of necrosis of the liver, and thin fibrous adhesions more or less
completely gluing together the contents of the cranial half of the abdomen, in-
cluding the diaphragm and the cranial half of the right kidney.
The necrosis as seen with the unaided eye involved one-fourth to one-half of the
liver in 2 instances, half to three-fourths in 13, and over three-fourths in the
remaining 2. Ascites was present in 4 rats, and pulmonary edema in 6. Micro-
scopically there were massive areas of hepatic necrosis, with the viable portions
of the liver generally showing a mixture of regenerative hyperplasia and (from the
incidental slight or moderate degree of inanition) a lesser degree of atrophy.
Varying amounts of loose to dense fibrous tissue were present around the large
areas of necrosis and irregularly in the viable portions of the liver. In the
meshes of the fibrous tissue were generally numerous foamy macrophages, and
smaller numbers of these macrophages were present in the sinusoids. The
macrophages, and to an equal or greater extent the hepatic cells, contained mod-
erate numbers of small basophilic granules of a type not previously seen in any of
our experimental animals of any species. Blood vessels both inside and outside
the liver in the vicinity of the large necrotic areas often contained thrombi,
sometimes organized and canalized; the vessels generally appeared to be parts of
the portal venous system, rarely of the hepatic arteries. Slight or moderate bile
duct proliferation in the liver was seen frequently. Macrophages containing
hemosiderin were generally present in the fibrous tissue gluing the cranial surface
of the liver to the diaphragm, and to a lesser extent around the large areas of
necrosis and elsewhere in the liver. Focal myocardial necrosis with varying de-
grees of replacement by fibrous tissue was frequent, but was usually slight in
degree in contrast to the more extensive examples noted after longer periods of
feeding, and at lower dosage levels. Nearly all the rats whose leg muscles were
sectioned showed focal necrosis of the muscle fibers, generally moderate in degree,
with more or less fibrous replacement. The lungs frequently contained foci of
large agranular foamy macrophages, tending to occur subpleurally and peribron-
chially and making up from 25 to 35% of the area of the micro-
scopic section. These macrophages, together with the somewhat less frequent
edema fluid and a small alveolar and septal cellular exudate which was chiefly
mononuclear (smaller and more solid macrophages, together with some poly-
morphonuclear leukocytes), made a rather characteristic picture in this and other
groups on the higher dosage levels. Basophilic granules similar to those in the
liver were present in renal glomerular and tubular epithelial cells, and in lesser
concentration in masses of foamy macrophages in the villi of the small intestine,
in scattered macrophages in the splenic pulp and lymph nodes, in the cells of the
adrenal cortex, in myocardial and pulmonary venous muscle cells, and in the
endothelial cells of arteries, chiefly those of the lung, heart, liver and spleen.
They were very rare in arterial muscle cells, which were frequently slightly en-

larged and contained weakly oxyphilic granules.   Thirteen spleens in this group were slightly enlarged; this enlargement was accounted for microscopically by a slight pulp hyperplasia plus the presence of the macrophages.

To aid in studying the pathogenesis of the changes resulting from quinacrine administration and to determine what fraction of the changes resulted from the moderate degree of inanition present, a special group of 24 rats, not forming a part of the 180 rats on the 2-year experiment, was divided into 2 groups, 12 rats receiving 800 ppm. quinacrine in the diet and 12 rats receiving no quinacrine but with each animal having its dietary intake restricted to that of its paired litter mate.   Because all of the rats in the first series had with one exception lived at least 13 weeks, it was intended to sacrifice 1 male and 1 female of the second series together with their paired feeding controls, at 3, 5, 7, 9, 11 and 13 weeks.   However, after the first 2 pairs had been sacrificed at 3 and 5 weeks respectively, the remaining 8 treated rats either died or were sacrificed in extremis at from 5 to 8 weeks.   A slightly faster rate of bringing the animals to the 800 ppm. level than that used for the first group may have been responsible for the earlier deaths. The microscopic changes were essentially identical with those described in the preceding paragraph, with even greater portions of the liver being necrotic in the last 8 treated rats.   The pair sacrificed at 3 weeks showed a slight to moderate hydropic degeneration of the liver, while the pair sacrificed at 5 weeks showed a moderate fatty degeneration.   Small numbers of foamy macrophages in the lung and small intestine had already made their appearance.   The male of each of the above pairs showed focal necrosis of the myocardium; leg muscles were negative. In the remaining animals the leg muscles showed focal necrosis similar in degree to that in the first group; hepatic necrosis and other histopathologic changes were also similar with the exception that the myocardium was slightly less damaged. The bone marrow of the rats receiving quinacrine was generally slightly hyperplastic while that of the paired feeding controls was slightly hypoplastic.   The treated rats showed slight reduction of new bone formation at the epiphyseal lines and sometimes slight osteoporosis beyond the changes of these types resulting in the control rats from reduced dietary intake.

Returning now to the main experiment, at the same dosage level of 800 ppm. but in a high protein diet, the first rat of the group of 18 was sacrificed because of poor condition after 21 weeks of feeding.   Twelve of the remaining 17 died or were sacrificed because of poor condition between the 26th and 32nd weeks, while the last rat died after 50 weeks.   The gross and microscopic changes in the liver were more variable than in the low protein group, some showing the extensive changes described above, others less, with almost no macroscopic change in some instances, and the microscopic lesions consisting of slight to moderate focal necrosis and additional minor changes.   In 9 of the 18 rats there were upper abdominal adhesions and macroscopic liver necrosis; the mean degree of these changes was less than in the low protein group.   Cardiac damage, however, was greater than in the low protein group.   Moderate dilatation was generally present, and in the microscopic sections there was usually marked myocardial fibrosis, best seen in the left ventricle.   Small numbers of muscle fibers showed recent

necrosis, which together with the appearance of variable age in the fibrous tissue indicated that the nature of the process was that of continuing necrosis of muscle fibers, small in number at any given time.   Nine rats showed a lesion not seen in the low protein group, namely, thrombus formation in the left atrium of the heart; in a tenth rat there was a mural thrombus in the left ventricle.   The atrial thrombi were usually 6 or 7 mm. in diameter and were whitish in color.   Microscopically there was usually slight peripheral organization in the thrombi. Ascites was not present in this group; edema of the lungs was noted in 9 animals and hydrothorax in 1.   The spleen was slightly enlarged in 7 animals.   Basophilic granulation, foamy macrophages, low grade pulmonary consolidation, focal necrosis of leg muscles, splenic and bone marrow hyperplasia and other changes were similar to those in the 800 ppm. low protein group.

At a dosage level of 400 ppm. in either a high or low protein diet, the majority of the rats had died or had been sacrificed because of poor condition at the end of 50 to 60 weeks of feeding.   There was little of the massive macroscopic necrosis of the liver seen in the 800 ppm. groups but the lesions were still of high grade, especially in the microscopic sections.   Whitish thrombi in the left cardiac atria of the same size as in the previous group were present in 7 rats of the high protein group, and in 2 of these ventricular thrombi were also present, once each in the right and left ventricles.   In the low protein group there were 6 atrial thrombi, plus left ventricular thrombi in 3 of these 6 rats.   Edematous lungs were noted 8 and 5 times respectively, and hydrothorax once in each group.   Ascites was seen only once, in a low protein rat other than that having hydrothorax.   Both groups showed the dilatation of the heart seen in the 800 ppm. high protein group, and in addition distinct hypertrophy was evident.   Only 1 rat in the high protein group showed a macroscopic area of necrosis in the liver, while these, together with upper abdominal adhesions, were seen 3 times in the low protein group. Of the remaining livers, about half were grossly negative, while the other half showed slight changes such as a nutmeg appearance, roughening of the surface, or slight disproportions and distortions of the architecture.   The spleen often showed a minimal enlargement, but definite enlargement was noted only once in the high and twice in the low protein group.   Microscopically, in both the high and low protein groups, approximately equal numbers of animals showed slight, moderate and marked liver damage, consisting chiefly of necrosis, with slight regenerative changes.   The necrosis was most often centrolobular, less often patchy, and only in a few instances in massive blocks.   Myocardial scarring (old and recent necrosis of muscle fibers with replacement fibrosis) was usually moderate in degree in the high protein group, while in the low protein group it was either moderate or marked.   Hypertrophy and rarefaction of the muscle fibers of the left ventricle and large pulmonary veins were frequent, but basophilic granules in the muscle fibers were rarely seen.   The atria and right ventricle of the heart showed the hypertrophy and rarefaction to a lesser degree than did the left ventricle; the same was true of the necrosis and scarring.   Basophilic granules in hepatic and renal epithelial cells and in macrophages in the liver, small

intestine and spleen were present in small to moderate numbers, showing a moderate reduction from the number present when 800 ppm. were fed. The granules were now rare in arterial endothelial cells. In general, leg muscles showed slight focal necrosis and/or fibrosis, and the bone marrow and splenic pulp were slightly hyperplastic. The lungs were about as described for the 800 ppm. level. The adrenal showed a mild degree of damage not seen at other levels; in about one-fourth of the animals the inner portion of the cortex had a very slight to moderate moth eaten appearance probably from previous damage with replacement by loose fibrous tissue, and in a few adrenals small foci of frank necrosis were present. Another change noted only at this level was the rather frequent presence of small amounts of pigment, chiefly nonferrous, in the renal convoluted tubular epithelium. In summary, in the 400 ppm. groups damage to the heart seemed to be as great a factor in causing death as was damage to the liver which is contrary to the findings in the groups on higher dosage. A high protein level in the diet had only a slight sparing effect on the development of anatomical changes and on mortality as compared to the marked effect seen when a diet containing 800 ppm. quinacrine was fed.

TABLE 4

Numbers of rats with microscopic changes of moderate or marked degree

GROUP	MICROSCOPICALLY EXAMINED	HEART	LIVER	KIDNEY
200 ppm.	23	9	7	4
100 ppm.	27	2	2	4
Control	16	0	0	0

At a dosage level of 200 ppm. the treated animals did not begin to die in greater number than their controls until after about 18 months of feeding. Microscopically, the visible changes at this level were slight. The viscera showed a slight yellow staining, and a minority of the livers had such changes as slight enlargement, slight roughening of the surface, a fine dark orange-and-red mottling, and some rounding of the edges. Pulmonary edema was present in 3 animals, 2 in the high protein group. In the one in the low protein group, which died after 20 months, hydrothorax and the only cardiac atrial thrombus at this level were also present. A few hearts were slightly dilated and hypertrophied and a few spleens very slightly enlarged. In the 100 ppm. groups the gross changes were on the whole very slight, although one rat on the low protein diet, evidently unusually susceptible, showed extensive hyperplasia and architectural distortion of the liver when sacrificed after 15 months. Microscopically, lesions attributable to quinacrine in the 200 and 100 ppm. groups, whether on a high or low protein diet, were essentially limited to the liver and heart and to a lesser extent the kidney. The mild kidney damage appeared late in the experimental period. The actual numbers of lesions graded as moderate or marked in the microscopically examined rats are given in table 4; slight or very slight degrees of most of these changes

were present in many of the control animals (as they are in any group of rats two years of age) and in more of the test animals. The cardiac lesion was focal myocardial fibrosis, greatest in the left ventricle, and the renal lesion consisted of focal cortical tubular atrophy and dilatation, the presence of hyaline tubular casts, and some glomerular atrophy. In these two organs the lesions caused by quinacrine at such low levels of dosage were not specific histologically and differed only in degree and numerical incidence from the "spontaneous" lesions in control animals of this species; this was not true for the liver. Lesions of the liver were never more than moderate in degree except in the one unusually susceptible animal and included (in addition to accentuation of the common "spontaneous" slight hepatic cell atrophy and bile duct proliferation seen in old rats) hepatic cell hyperplasia, disarrangement of normal architecture, centrolobular necrosis, and rarely focal vacuolar degeneration of hepatic cells. The basophilic granules and macrophages regularly seen in the 400 ppm. groups were seen infrequently and in small numbers at the 200 ppm. level and only once at 100 ppm. Slight focal necrosis and/or fibrosis of leg muscles was seen infrequently at 200 ppm. and not at 100 ppm.; slight hyperplasia of the splenic pulp was seen occasionally in both groups, and the adrenal and bone marrow were negative in both.

The thyroid deserves a word of mention since we were unable to find a reference to it in previous reports. Twenty-one thyroids scattered among the various treated groups were examined microscopically and the only hyperplasia noted was a minimal or questionable one in three glands. Another gland showed a marked depletion of colloid, interpreted as recent. These four glands were all in rats on a high protein diet at levels of 200 and 400 ppm. quinacrine; otherwise, the glands were similar to those of the controls. Parathyroids were included in most of the thyroid sections and were all negative. An unpublished study of a relatively large series of dogs given 2 and 5 mgm./kgm./day of quinacrine for 2 years showed only minimal changes in the thyroid (16).

The following remarks on the pathological changes apply to most or all of the rats in this study. The large areas of necrosis in the liver had an infarct-like appearance and as stated thrombi were frequently found in microscopic sections. However, certain groups showed rather frequent examples of multiple small foci of necrosis or larger areas of an earlier or lower grade of necrosis, without the presence of thrombi. Considering the entire series, it was our impression that in general thrombosis was secondary to the attainment of a large area of high grade necrosis, or at least that such an area was not primarily an infarct suddenly following thrombosis. Although exceptions were not uncommon, the right side of the liver was more susceptible to necrosis; Seigel and Mushett (1) noted the same tendency. The clustered foamy macrophages in the lung were larger than the foamy macrophages located elsewhere, were easily ruptured, and contained very few basophilic granules. The macrophages, endothelial cells and muscle cells containing basophilic granules frequently contained in addition poorly defined oxyphilic and fewer neutrophilic granules, whether these represented additional types of ingested material or were transitional forms of the basophilic

material is not known. In the small intestine multiple levels were frequently sectioned, and showed an increasing gradient of macrophages and basophilic granules from the upper to the lower end; these were not seen in the stomach or colon. In spite of their frequent presence in renal glomerular epithelial cells, basophilic granules were never noted in the adjacent endothelial cells. Among the renal tubular epithelial cells, basophilic granulation and its frequent accompaniment of hypertrophy and some rarefaction of the cell were essentially limited to the distal portions of the looped tubules and less often the proximal thin portions; they were rarely seen in convoluted or collecting tubules. The granules had the same appearance following Bouin or Zenker fixation as they did after formalin. The granules were not metachromatic when stained with toluidine blue, and they did not stand out when an unstained paraffin section or a smear of liver was examined with polarized light. Granules often remained basophilic in necrotic hepatic cells. We assume that these basophilic granules, colorless in unstained sections, are some quinacrine-protein compound, but we have no exact knowledge of their nature. The macrophages containing them often contained sudanophilic material in the liver, but not in other locations; the pulmonary macrophages were not sudanophilic. In the most severe examples of myocardial fibrosis, there was about the same amount of fibrous as of muscular tissue in the left ventricular wall. The cardiac valves never showed damage. In the foci of myocardial necrosis of short duration (800 ppm. low protein group) there was often slight calcification. Ascites, hydrothorax and pulmonary edema were more frequent in the animals found dead than in those sacrificed because of poor condition. The mechanism of formation and the chemical characteristics of the fluids were not investigated. A chronic passive congestion of the liver, often accompanying such fluid formation in man, was not seen. Voluntary muscles other than those of the leg were occasionally sectioned, and showed the same focal necrotizing changes as did the leg muscles. The adrenals of the rats on the higher dosage levels were enlarged, as noted by Seigel and Mushett (1) and by Barlow et al. (9). Splenic hemosiderosis was not a result of quinacrine administration; the treated rats had no more than the moderate amount of pigment present in the controls. Neither was there an excess of the usual juxtamedullary cortical pigment in the adrenal. As already noted, hemosiderin was present to a certain extent in the liver; lymph node hemosiderosis varied as in untreated rats from almost none to moderate, and a small or very small amount of hemosiderin was often present in the deeper portion of the lamina propria of the small intestine, underneath the foamy macrophages in the villi. The uteri and ovaries of 7 rats scattered among the various treated groups were sectioned and showed nothing of note; the same was true of 4 brains and 4 each of submaxillary and sublingual salivary glands. Testicular atrophy from quinacrine was only slight when the factors of inanition and of late "spontaneous" atrophy in the controls were considered; a possible slight degree of atrophy of the pancreas belongs in the same category. In both treated and control groups on a low protein diet, calcified tubular casts around the renal corticomedullary junction were frequently seen, while they were scarce in the high protein groups. These renal calcified tubular

casts are a typical result of a low protein diet, with accentuation by inanition, in our rats, and there is surprisingly little tubular atrophy proximal to these casts.

In view of the destructive effect of quinacrine on the liver, its possibilities as a cirrhogenic and carcinogenic agent in prolonged administration must be considered. It is true that several of the rats discussed in this paper had livers that both microscopically and grossly showed nodularity and portal fibrosis to such an extent that were hepatic cirrhosis the primary object of this study these livers would have been considered to show positive results. On the other hand, these results did not appear in the rats on low dosage levels and living for 2 years. Only 1 liver tumor was seen in the group, an adenoma 2.2 x 2.0 x 1.1 cm. composed of hepatic cells, in a rat that received 100 ppm. in a high protein diet for 2 years; this is a normal incidence of this type of tumor in our rats.

PART II. THE EFFECT OF STARTING AGE ON THE TOXICITY OF QUINACRINE. Ten litters of 4 male rats each were placed on an adequate diet containing 800 ppm. of quinacrine and allowed to remain on this ration until they died. A rat from each of the litters was placed on the quinacrine-containing diets at weekly intervals from 3 weeks of age until all rats had been placed on their diets.

An analysis of the data showed that there was no significant difference in mortality in the 4 groups of rats. The older and therefore the larger rats died in about the same number of days on the experimental diet as the younger rats. An analysis of variance, however, brought out the fact that there was a significant difference in the mortality rate between litters. A rat in any given group which resisted the toxic action of quinacrine for a longer time had litter mates in the other 3 groups which also resisted the toxic action of quinacrine.

Discussion. The many toxic effects produced by quinacrine in these experiments compels a consideration of the relationship of the dosage received by the rats and the human dose in the treatment of malaria. According to Circular Letter No. 22, Office of the Surgeon General, War Department (17) the therapeutic dose for malaria in man is 300 mgm. given daily for a week and the prophylactic dose is 200 mgm. given semi-weekly. Chart 1 shows the calculated quinacrine intake per day in terms of the body weight of the rat. These values were calculated from the weekly food intake of the rats at the various monthly intervals. The quinacrine intake of the rats at any given dosage level decreased rapidly during the first 2 months and then became almost constant as shown by the straight line (Chart 1) at about 6 months on the experimental diet. This fact is accounted for by the change in the growth rate of the rats from the fast growing period to the plateau period, while at the same time their daily food intake remained almost constant. Part of the increased toxicity observed in the rats on the low protein diet is undoubtedly caused by the greater amount of quinacrine consumed than by rats with a similar dosage level on the high protein diet. As shown in the chart the dosage of 100 ppm. of quinacrine corresponds to about 4 mgm./kgm. of body weight per day for the rat. The dosage level is the borderline at which slight damage may occur to some rats given quinacrine for 2 years and to others no harm occurs. Taking the length of time into consideration, therefore, the amount of quinacrine that will produce toxic effects in rats is above the therapeutic or prophylactic dose for man.
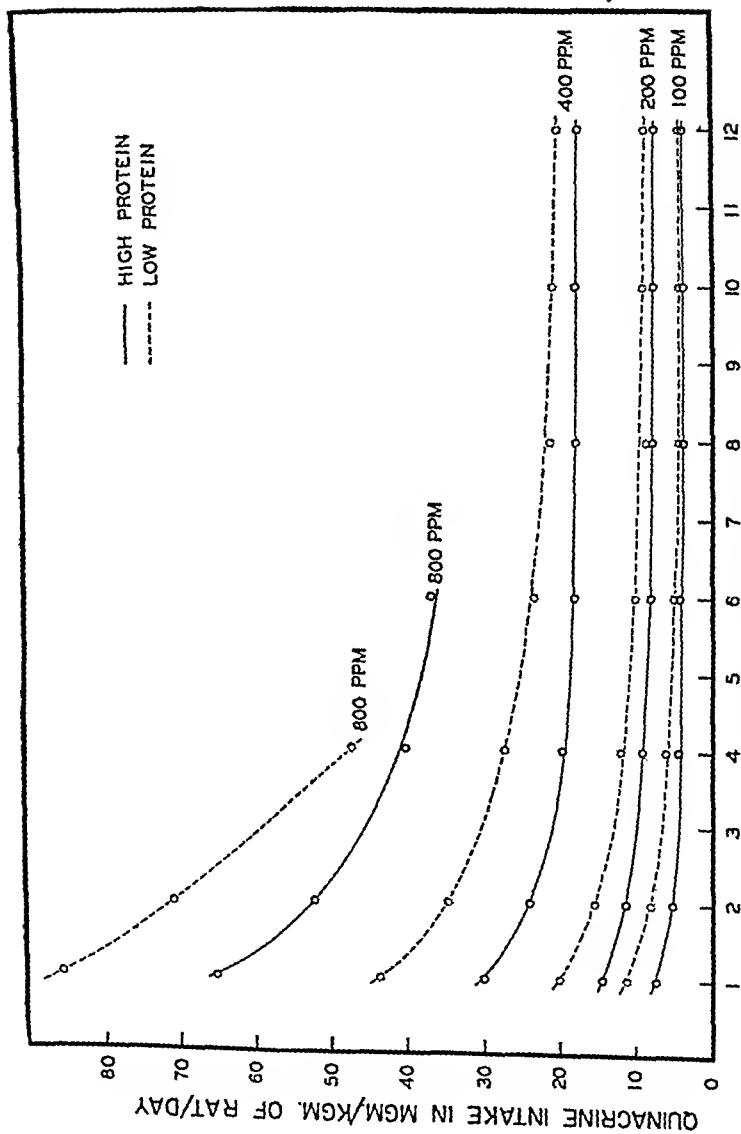
CHART 1. CALCULATED QUINACRINE INTAKE IN MGM./KGM. OF BODY WEIGHT IN RATS RECEIVING VARIOUS LEVELS OF QUINACRINE IN THE DIET

In another experiment, involving 59 rats, 400 and 800 ppm. of quinacrine in an adequate diet produced the same effects as those reported above for the rats at similar concentrations in a high protein diet.

SUMMARY AND CONCLUSIONS

1. Quinacrine at concentrations of 100 ppm. or more in the diet of rats produces toxic effects. At the 100 ppm. level the effects are slight, at higher levels they become progressively more severe.

2. On the low protein diet there was a significant retardation of growth at all concentrations of quinacrine used in this experiment; however, on the high protein diet there was no significant retardation below the 200 ppm. level. Because of a difference in food intake the rats on a low protein diet consumed more quinacrine per kgm. of body weight than those with a similar dosage level on a high protein diet.

3. A concentration of 800 ppm. of quinacrine produces early death in rats and one of 400 ppm. significantly increases the death rate; lower dosages do not affect mortality significantly.

4. The outstanding hematological change is a leukocytosis. This is marked in the groups on 800 ppm., less striking in the groups on 400 ppm. and scarcely noticeable in those on 200 ppm. of quinacrine. There is an increase in the hemoglobin concentration and erythrocyte counts in the rats on the 800 ppm. of quinacrine.

5. At a dosage level of 800 ppm. of quinacrine in the diet, any small group of our rats showed all and any individual rat showed most of the following changes: Generalized yellow discoloration; high grade necrosis of the liver; regenerative changes in the remaining viable liver; upper abdominal adhesions; focal necrosis and/or fibrosis of the myocardium and voluntary muscles; basophilic granules in hepatic, renal and other cells; foamy macrophages with or without similar basophilic granules in several locations; slight hyperplasia of the splenic pulp and bone marrow; and a low grade pulmonary consolidation.

6. At a dosage level of 800 ppm. a high level of protein in the diet considerably delayed and reduced the severity of liver damage as compared to that seen with a low level of protein; at 400 ppm. this delay and reduction was only slight, and at lower levels it was not apparent.

7. At 400 ppm. and lower levels of quinacrine, myocardial fibrosis and other cardiac changes, a more cumulative type of damage than that in the liver, became a factor equal to or greater than liver damage in causing the death of the animal.

8. After 18 to 24 months of feeding, a longer period than previously reported, rats on 200 ppm. of quinacrine in either a low or high protein diet frequently showed distinct anatomical lesions as a result of the medication. Even at 100 ppm. there was a small amount of damage.

9. The percentage of rats in which quinacrine caused cirrhosis of the liver was so small that we do not class quinacrine as a distinctly cirrhogenic agent. Only 1 liver tumor was seen, an adenoma which was probably incidental.

10. The age of young rats at the beginning of the experimental period had no

effect on the toxicity of quinacrine. There was found, however, a significant effect between different litters of rats. This fact demonstrates the importance of choosing litter mates for chronic feeding experiments.

11. In relation to the body weight of the rat the lowest dosage of quinacrine which produced slight toxic effects in some animals corresponds to approximately 4 mgm./kgm./day for 2 years.

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THE ANTIDIURETIC ACTION OF BARBITURATES (PHENO-BARBITAL, AMYTAL, PENTOBARBITAL) AND THE MECHANISM INVOLVED IN THIS ACTION[1,2]

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In a previous paper (1) it was shown that morphine inhibits water diuresis and that this action is dependent upon a functioning neurohypophysis. It was observed that morphine produces no antidiuretic action in those dogs in which the entire neurohypophysis has been destroyed with resulting permanent diabetes insipidus.

Since it was shown that morphine does not potentiate the antidiuretic action of the circulating posterior pituitary hormone, it was concluded that morphine brings about the liberation of the antidiuretic hormone by acting on the hypothalamico-hypophysial system.

In view of these findings, it seemed worthwhile to investigate whether other drugs acting on the central nervous system produce a similar action. We decided to study first the barbiturate group. The literature dealing with the effect of the barbiturates on water diuresis and kidney function is contradictory and confusing. Different investigators worked on a variety of species, used different barbiturates and diverse methods; many of the experiments were inadequately controlled. Table 1 briefly summarizes the work done on this subject so far.

The purpose of our investigation was 1) to determine whether the barbiturates inhibit water diuresis in adequately controlled experiments and, if they produce an antidiuretic effect, 2) to determine the mechanism of this action.

METHODS. All of the experiments were carried out on female dogs, 157 normal, 10 neurohypophysectomized (diabetes insipidus), and 6 hypophysectomized. These animals were kept on a constant diet (for composition see earlier paper) (1), were fed once a day about 17–18 hours preceding the commencement of the experiment and were therefore in the postabsorptive state throughout the experiment.

The experimental procedure followed was in principle the same as that described in the earlier work. Each experiment started with the administration of 40 cc /kgm. of tap water by stomach tube (first water). Three hours later the bladder was emptied by catheterization, the urine discarded, and either the same amount of water (40 cc /kgm.) was given by stomach tube or 25 cc./kgm. by slow intravenous infusion (second water). The animal was then placed in a metabolism cage. Three hours after the administration of the second dose of water the bladder was again emptied by catheterization and the urine excreted during this second three hour period collected quantitatively. The first dose of water

TABLE 1

DRUG	AUTHOR	SPECIES	DOSE AND METHOD OF ADMINISTRATION	WATER DIURESIS
Phenobarbital sodium	Molitor & Pick, 1927 (2)	Dog	0.008–0.024 gram/kgm. by stomach tube	Decreased
	Buschke, 1928 (3)	Rabbit	0.08 gram/kgm. subcutaneously	Decreased only in 2 out of 7
	Kugel, 1929 (4)	Rabbit	0.12–0.2 gram subcutaneously	Decreased
	Bonsmann, 1930 (5)	Dog	0.05 gram/kgm. by stomach or duodenal tube	Decreased
Isopral	Molitor & Pick, 1927 (2)	Rabbit	0.11–0.25 gram/kgm. by stomach tube, subcutaneously or intravenously	Decreased
Amytal sodium	Fee, 1928 (6)	Dog	By mouth	Decreased
	Ogden, 1930 (7)	Dog	0.05 gram/kgm. intraperitoneally	Decreased
Sandoptal	Kugel, 1929 (4)	Rabbit	0.2 gram by stomach tube	Increased
	Bonsmann, 1930 (5)	Dog	0.04–0.08 gram/kgm. by duodenal tube	Decreased
Barbital sodium	Frey & Kumpiess, 1914 (8)	Human (normal & diabetes insipidus)	0.5 gram veronal + 1 gram trional by mouth	Decreased
	Kugel, 1929 (4)	Rabbit	0.1–0.2 gram/kgm. subcutaneously	Unchanged or increased
	Bonsmann, 1930 (5)	Dog	0.1 gram/kgm. by duodenal tube	In 33% decreased, in 67% no change
Phenobarbital + Barbital sodium	Kugel, 1929 (4)	Rabbit	0.12–0.2 gram phenobarbital + 0.1 gram barbital sodium subcutaneously	Unchanged or increased
Pentothal sodium	Silvette, 1942 (9)	Rat	4–6 mgm./100 gram body wt. intraperitoneally	Decreased—0.2% NaCl given intraperitoneally instead of water.
Pentobarbital sodium	Mylon, Winternitz, de Sütö-Nagy, 1943 (10)	Dog	30 mgm./kgm. intravenously	Suppression of renal function in some animals.

TABLE 1—*Concluded*

DRUG	AUTHOR	SPECIES	DOSE AND METHOD OF ADMINISTRATION	WATER DIURESIS
Pentobarbital sodium—*Continued*	Mylon, Winternitz, de Sütő-Nagy, 1943 (10)	Dog	25 mgm./kgm. intravenously	No change in renal function.
	Corcoran & Page, 1943 (11)	Dog	30 mgm./kgm. intraperitoneally	Renal function, as measured by diodrast & inulin clearance, normal; urine flow less in 4 out of 8 instances; temporary failure of renal function in 3 out of 7.

was given only to bring the animal into water equilibrium. The excretion of the second dose of water was the basis of the study of the drug action, since it has been shown that this dose of water is excreted quantitatively in normal dogs.

The drug to be studied was injected intravenously. If the second water was given by stomach tube, the drug was administered 40 minutes following the water administration in order to allow time for the absorption of the water from the gastrointestinal tract before introducing the drug. The urine which was excreted during this 40 minute interval was collected quantitatively and subtracted from the amount of the second dose of water administered when the percentual recovery was calculated, since its excretion was obviously uninfluenced by the drug. If, on the other hand, the water was infused intravenously, the drug was administered just before the water infusion was begun. By using this technique, the period during which the urine was collected was 2 hours and 20 minutes in the former case and 3 hours in the latter. In order to avoid the development of any possible tolerance, an interval of more than 12 days was allowed between successive administrations of the barbiturates. For explanation and further details of the experimental technique see earlier paper (1).

The following barbiturates were used: phenobarbital sodium, amytal sodium[1], pentobarbital sodium.

RESULTS. *The effect of phenobarbital on water diuresis in normal, diabetes insipidus and hypophysectomized dogs.* In the first series of experiments on normal dogs the second dose of water was given by stomach tube and 40 minutes later the drug was injected intravenously. It was found that phenobarbital sodium in doses smaller than 0.035–0.040 gram/kgm. had no consistent effect on water diuresis. In doses of 0.04 gram/kgm. water diuresis was inhibited in all 42 experiments done on 21 dogs. As can be seen in table 2, the urine excreted during the 2 hour 20 minute period following the administration of 0.04 gram/kgm. of phenobarbital sodium was in average $47\% \pm 8.2\%$ as compared with $90\% \pm 7.6\%$ when no drug was given. Since it was shown that the absorption of the second dose of water from the gastrointestinal tract

was completed within 40 minutes, it is apparent that phenobarbital could not have interfered with this absorption. The decreased excretion of water can only be attributed then to an inhibition of the excretory mechanism. Nevertheless for further corroboration, in a second series of experiments the second dose of water was infused slowly into a large vein and the drug was given before the infusion was started. Phenobarbital inhibited water diuresis in these experiments to the same degree as.it did in those in which the water was given by stomach tube (table 2).

Immediately following the injection of this amount of phenobarbital the animals usually showed some excitement and became ataxic. If left undis-

TABLE 2

The effect of phenobarbital on water excretion after ingestion or intravenous infusion of water in normal dogs

All experiments started 17–18 hours after last feeding. In each experiment 40 cc./kgm. water was given by stomach (first water). Three hours later the bladder was emptied by catheterization and then the second dose of water given.

NUMBER OF DOGS	NUMBER OF EXPERI- MENTS	SECOND WATER		DOSE OF PHENOBARBITAL SODIUM* GRAM/KGM. INTRAVENOUSLY	URINE DURING 3 HRS. IN % OF SECOND WATER, MEAN AND MEAN DEVIATION	EFFECT ON NERVOUS SYSTEM
		Given by	Amount cc./kgm.			
38	108	Stomach	40		90 ±7.6	
14	14	Vein	25		111 ±17.7	
21	42	Stomach	40	0.04	47 ±8.2	Excitement, ataxia, followed by sleep
10	10	Vein	25	0.04	45 ±8.4	Excitement, ataxia, followed by sleep
12	12	Stomach	40	0.08	43 ±6.0	Light or full anesthesia
6	6	Stomach	40	0.105–0.110	19 ±1.1	Deep anesthesia

* Time of phenobarbital administration: 40 minutes after the second dose of water was administered, when this was given by stomach or a few minutes before the intravenous infusion of the second dose of water was started. In the former case the urine excreted in the interval between the administration of the second dose of water and the injection of phenobarbital was quantitatively collected and deducted from the total amount of second water in calculating the percentual recovery.

turbed, they fell asleep within a few minutes and remained so for several hours. It was easy to arouse them and while they were able to walk, they showed some incoordination.

0.08 gram/kgm. of phenobarbital, which produced light or full anesthesia usually while the intravenous injection was being made, had about the same degree of antidiuretic effect as the smallest effective dose. Still larger doses (0.105–0.110 gram/kgm. given intravenously), which produced deep surgical anesthesia, exerted a more marked effect on water diuresis (table 2). This anesthesia lasted for about 24 hours, and 48 hours after the injection the animals were still unable to stand.

Thus, phenobarbital in proper doses was effective in inhibiting water diuresis in every normal animal studied. This action was similar to that of morphine in its constancy, but not so marked in its degree, even when phenobarbital was given in full anesthetic doses. Since this antidiuretic action of phenobarbital was established, the question arose as to whether the neurohypophysis was involved in the same way as it is in the case of morphine. In order to answer this question, the effect of phenobarbital sodium on water diuresis was studied in dogs in which the entire neurohypophysis was destroyed.

For this purpose the animals were prepared in the following way: a high hypophysial stalk section was done and in addition the anterior part of the hypothalamus was injured. This resulted in loss of function and degeneration of the entire neurohypophysis (infundibular process, infundibular stem and

TABLE 3

The effect of 1) phenobarbital, 2) amytal and 3) pentobarbital on water excretion after ingestion of water in diabetes insipidus dogs

All experiments started 17–18 hours after last feeding. Experimental procedure and time of drug administration as in table 2.

DOG NUMBER	No drug, mean and deviation	Phenobarbital sodium intravenously		Amytal sodium intravenously		Pentobarbital sodium intravenously	
		0.040 g /kgm.	0.080 g /kgm.	0.025 g /kgm.	0.050 g /kgm.	0.016 g./kgm.	0.032 g /kgm.
DI 2	71 ±7.3	79	72	75	70	82	78
DI 6	80 ±9.6	89	81	100	79	81	78
DI 13	87 ±23.0	90	85	69	90	117	84
DI 15	70 ±5.5	81	74	83	76	83	73
DI 17	92 ±13.1	109	92	120	89	116	90
DI 12	71 ±6.4	80	75	76	70	82	78
DI 16	76 ±5.8	72	74	82	77	86	81
DI 18	71 ±6.7	81	74	84	80	85	77
DI 7	78 ±7.4	91	86	86	87	92	90
DI 14	67 ±5.8	76	74	75	72	81	78

median eminence). These animals developed permanent diabetes insipidus—their daily water exchange was from 3500 to 7000 cc. They had a normally functioning anterior pituitary and some of these animals lived for over four years. For further details see paper on morphine antidiuresis (1).

As was shown in earlier work from this laboratory, diabetes insipidus dogs excrete ingested water somewhat more slowly than do normal animals. It was found that the amount of urine excreted by a large number of diabetes insipidus dogs in the 3 hours following the ingestion of water was in average 73% as compared to the average of 90% in normal dogs. Phenobarbital sodium given intravenously either in doses of 0.04 gram/kgm. or in full anesthetic doses did not inhibit water diuresis in dogs with diabetes insipidus (table 3). The results were the same whether the water was given by stomach tube and the

drug administered 40 minutes later or whether the drug was given first and the water infused intravenously.

In contrast to these results are those which we obtained with hypophysectomized dogs. In these animals, the entire adenohypophysis (pars distalis, pars intermedia and pars tuberalis) and the infundibular process (neural lobe) were removed. These animals had a low postabsorptive blood sugar level, were sensitive to insulin, went into hypoglycemic crises on fasting, or sometimes spontaneously, but had no permanent diabetes insipidus. The median eminence was not destroyed, it was still producing antidiuretic hormone.

These hypophysectomized animals, due to their diminished blood pressure and decreased renal blood flow with resultant decreased glomerular filtration, excreted ingested water even more slowly than did those dogs with diabetes insipidus. The control recovery of urine during the 3 hour period following the administration of the second dose of water was an average of 52%. When

TABLE 4
The effect of phenobarbital on water excretion after ingestion of water in hypophysectomized dogs

All experiments started 17–18 hours after last feeding. Experimental procedure and time of drug administration as in table 2.

DOG NUMBER	URINE DURING 3 HOURS IN % OF WATER GIVEN	
	No drug, mean and deviation	Phenobarbital sodium intravenously 0.040 g /kgm.
H 5	59 ±8 9	34
H 6	56 ±8 7	31
H 7	50 ±7 1	29
H 8	51 ±2 2	32
H 9	48 ±4 9	29
H 10	49 ±3 8	26

phenobarbital was given, water diuresis was inhibited in these animals to about the same degree as in normal animals (table 4).

Having obtained these results with phenobarbital, we proceeded to study the effect of amytal on water diuresis.

The effect of amytal sodium on water diuresis in normal and diabetes insipidus dogs. In our study of the effect of amytal on water diuresis, two series of experiments were conducted on normal animals. In the first, 0.025 gram/kgm. of amytal sodium (one-half of the full anesthetic dose) was administered intravenously. This dose inhibited water diuresis in only 14 dogs out of a total of 32 (table 5), the degree of this antidiuretic effect (49% ± 3.8%) was approximately the same as that obtained with 0.04 gram/kgm. of phenobarbital (47% ± 8 2%). The remaining 18 dogs showed no antidiuretic response to this dose of amytal; they excreted 81% ± 9.9% of the ingested water. In the second series of experiments, the full anesthetic dose (0.050 gram/kgm.) was given and while this produced an inhibition of water diuresis in 18 dogs out of a total of 22,

the degree of inhibition was still the same as that of the smaller dose. There were still, however, four dogs in the group which did not respond with an antidiuretic effect.

In order to elucidate the mechanism of this antidiuretic action, further experiments were done on dogs with diabetes insipidus. The drug was administered in the same doses as were used in the experiments on normal dogs and it was found that amytal sodium in either dose did not inhibit water diuresis in these diabetes insipidus dogs (table 3).

The effects observed immediately following the injection of the smaller dose consisted of excitement, muscular incoordination, and eventually sleep. The larger dose produced anesthesia usually while the injection was being made

TABLE 5

The effect of 1) amytal and 2) pentobarbital on water excretion after ingestion of water in normal dogs

All experiments started 17–18 hours after last feeding. Experimental procedure and time of drug administration as in table 2.

NUMBER OF DOGS	NUMBER OF EXPERIMENTS	DRUG*	DOSE OF DRUG	URINE DURING 3 HOURS IN % OF WATER GIVEN, MEAN AND DEVIATION	EFFECT ON NERVOUS SYSTEM
			g /kgm		
32 {14	14	Amytal sodium	0.025	49 ±3.8	Excitement, ataxia, followed by sleep
18	18			81 ±9.9	Excitement, ataxia, followed by sleep
22 {18	18	Amytal sodium	0.050	46 ±8 2	Anesthesia
4	4			83 ±8.5	Anesthesia
24 {12	12	Pentobarbital sodium	0.016	51 ±5.3	Excitement, ataxia, followed by sleep
12	12			83 ±8.0	Excitement, ataxia, followed by sleep
30 {20	20	Pentobarbital sodium	0 032	53 ±6.5	Anesthesia
10	10			76 ±4.9	Anesthesia

* The drug was administered intravenously in every experiment.

and this anesthesia lasted for several hours. These effects developed in all animals regardless of whether or not the drug inhibited water diuresis.

This striking result namely, that amytal even when given in large doses did not inhibit water diuresis in all animals, suggested the study of still other barbiturates and so we chose to investigate the effect of pentobarbital on water diuresis.

The effect of pentobarbital on water diuresis in normal and diabetes insipidus dogs. The experimental procedure followed was the same as that employed previously in our study of phenobarbital and amytal. To one series of dogs the drug was administered in an amount equivalent to one-half of the anesthetic dose and to another the full anesthetic dose was given. The smaller dose

(0.016 gram/kgm.) inhibited the excretion of water in 50% of the animals studied and the degree of this inhibition was about the same as that produced by the smaller dose of either phenobarbital or amytal (table 5). In full anesthetic doses (0.032 gram/kgm.) pentobarbital had an antidiuretic effect in 66% of the animals, but the inhibition of water diuresis was not more marked than that obtained with the smaller dose (table 5). Neither dose of pentobarbital was effective in inhibiting water diuresis in diabetes insipidus dogs (table 3).

The smaller dose of pentobarbital produced sleep after a transient excitement, while the larger dose brought about immediate anesthesia which usually lasted from three to four hours. Again these effects occurred regardless of the effect on water diuresis.

The constancy of the effect of pentobarbital on water diuresis in normal dogs upon repeated administration of the drug. Since it was observed in our studies both

TABLE 6

The effect of repeated administrations of pentobarbital sodium on water excretion after ingestion of water in normal dogs

Three experiments were carried out on each dog, an interval of at least two weeks being left between experiments. All experiments started 17–18 hours after last feeding. Experimental procedure and time of drug administration as in table 2. The drug was administered intravenously.

NUMBER OF DOGS	EXPERIMENT NO.		
	1	2	3
3	−	−	−
4	+	+	+
2	−	+	−
1	−	−	+
1	+	−	−
1	+	−	+
1	+	+	−

+ represents antidiuretic effect.
− represents no antidiuretic effect.

of amytal and of pentobarbital that a certain percentage of the animals responded with antidiuresis, while the remainder had an unchanged diuresis, the question arose as to whether this response was a constant phenomenon of an individual animal or whether it varied upon repeated administrations of the drug.

On a series of dogs therefore, three experiments were carried out, care being taken to leave a time interval of at least two weeks between the administrations of pentobarbital sodium. Table 6 summarizes the results of these experiments. As can be seen, out of a total of 13, three dogs never responded with an antidiuretic effect, four dogs showed a consistent antidiuretic effect and the remaining six showed varying responses. In this latter group no definite pattern can be recognized. Two dogs gave an antidiuretic response only in the second experiment, one dog only in the third experiment and, another dog only in the

first experiment. The remaining two dogs gave two positive responses—one of these gave it on the first and second occasions and the other dog gave it on the first and third occasions.

From these results, it is evident then that the response of each individual animal to pentobarbital is not constant, but varies from one occasion to another.

It was of interest to learn whether morphine would exert an antidiuretic effect on those dogs in which pentobarbital did not influence diuresis. Therefore, to those dogs in which pentobarbital never produced an antidiuretic effect or produced it only on the first occasion that the drug was administered, morphine was given (5 mgm./kgm. intravenously) following the same experimental procedure as in the case of pentobarbital. In all these dogs morphine produced the typical, marked antidiuretic effect. The amounts of urine excreted were 1%, 3%, 6%, 7%.

The effect of phenobarbital on saline diuresis in normal dogs. Thus far we have discussed the effect of the barbiturates on water diuresis only. At this point we would like to consider their effect on saline diuresis.

In a series of normal dogs, 40 minutes after the administration of the second dose of water by stomach tube, a 20% sodium chloride solution was infused slowly into the jugular veins in a quantity which if mixed with the second water would have made the latter an isotonic sodium chloride solution. Fifty mgm./kgm. of phenobarbital sodium were injected intravenously either immediately before or after the infusion of the sodium chloride solution. Phenobarbital sodium was the barbiturate used in these experiments since, as was shown above, it was the barbiturate which inhibited water diuresis in every normal animal. The effects of phenobarbital on sodium chloride diuresis were not uniform. In some animals there was no antidiuretic effect whatsoever, while in others the antidiuretic effect was as marked as in the case of water diuresis. The percentual recoveries of urine during the experimental period were as follows: 112, 89, 80, 65, 63, 51, 39, 36. Increasing the dose of phenobarbital to 103 mgm./kgm. did not alter the results in any way. In a control series in which the same procedure was followed but phenobarbital omitted, the percentual recoveries of urine were about 100.

In another series of experiments, sodium chloride was replaced by sodium sulfate since this latter substance is not reabsorbed by the tubules to any considerable degree when its plasma level is abnormally elevated. For this reason it was considered more suitable for investigating whether or not phenobarbital could inhibit the diuresis produced by a salt solution. A 35% solution of anhydrous sodium sulfate was infused in amounts which if added to the second dose of water would have made it an isotonic solution (2%). Phenobarbital was not able to inhibit diuresis in any of these experiments, the percentual recoveries of urine varying from 93–119. When the amount of infused sodium sulfate was increased to such an extent that the second water became a 3% solution, the percentual recoveries of urine were much larger—168, 169.

In all of these saline diuresis experiments, the typical effects of phenobarbital on the nervous system were manifest.

It is apparent then that the effect of phenobarbital on saline diuresis is determined 1) by the physiological characteristics of the salt, 2) by the concentration of the salt, 3) in the case of sodium chloride, by individual animal differences. Thus, we may conclude that an osmotic diuretic can antagonize the antidiuretic action of phenobarbital on water diuresis.

DISCUSSION. One of the most striking observations made in this study was that the various barbiturates differ in their effects on water diuresis. Whereas phenobarbital inhibited water diuresis in every normal animal, amytal and pentobarbital did so only in some. However, this antidiuretic effect when produced (by either a small or a full anesthetic dose) was never so marked as that produced by a small dose of morphine. Nevertheless, the mechanism of this action is similar to that of morphine, in that the neurohypophysis is involved in both. Evidence for this is that neurohypophysectomized animals failed to respond to the barbiturates with an antidiuresis. Still further evidence that the antidiuretic hormone of the neurohypophysis is responsible for this action is the fact that in normal animals the barbiturates influence saline diuresis in a manner similar to pituitrin, that is, saline diuresis is either entirely uninhibited or inhibited to a lesser degree than water diuresis, depending upon the physiological properties and the concentration of the salt solution infused intravenously.

The varying responses of an individual animal to repeated administrations of pentobarbital may be due to a changing state of the neurohypophysis which, however, is not a factor in the case of morphine since the latter seems to represent a stronger and always effective stimulus.

Silvette, in his paper "The Mechanism of Pentothal Sodium Antidiuresis" (9) challenged our results published in a preliminary note (12). He based his criticism on the following experimental findings:

"A number of male rats were completely hypophysectomized . . . and were maintained after the operation for three to four weeks until the initial polyuria was succeeded by an essentially normal water intake and urine output. At this time extensive atrophy of the gonads was evident, indicating complete removal of the pituitary. . . . In these long-surviving, completely hypophysectomized animals, which had passed the polyuric state, the injection of pentothal sodium had the same antidiuretic effect as in normals. This indicates that the hypothalamicohypophyseal system is not involved in the production of pentothal sodium oliguria in rats."

These results obtained by Silvette do not justify his conclusion, for he confuses the criteria for the absence of the adenohypophysis with the criteria for the absence of the neurohypophysis. The fact that his animals had extensive atrophy of the gonads merely indicates a total or subtotal removal of the adenohypophysis (no histological evidence is given for the complete removal of the adenohypophysis), but certainly gives no evidence as to the condition of the neurohypophysis. Furthermore, the fact that his animals had only a temporary polyuria and had a normal water exchange at the time of the experiment, is definite evidence that at least part of the neurohypophysis was present and functioning and therefore, the hypothalamico-hypophysial system could have been

stimulated by the drug, resulting in an increased secretion of the antidiuretic hormone. As a matter of fact, Silvette's results are not in contradiction, but rather in fullest agreement with our findings. As was pointed out already in our preliminary papers (13, 14) hypophysectomized animals without permanent polyuria respond to morphine and the barbiturates with an inhibition of water diuresis, whereas neurohypophysectomized animals with permanent diabetes insipidus show no inhibition of water diuresis when these drugs are administered. The animals studied by Silvette correspond to those in the former group.

Corcoran and Page (11) in their study of the effect of pentobarbital sodium on renal function in dogs found lower rates of urine flow in only four out of eight instances when the drug was given in anesthetic doses. However, their experiments do not reveal the true effect of the anesthetic on urine flow, since in their clearance experiments the intravenously infused diodrast and inulin were dissolved in physiological saline containing 2% of sodium sulfate. As was mentioned above the intravenous administration of a salt solution can either completely inhibit or diminish the antidiuretic action of the barbiturates just as it can inhibit the pituitrin antidiuresis.

In this connection, we would like to emphasize that studies in which clearance methods are employed in investigating the effect of anesthetics on kidney function do not reveal the true effect of the anesthetic on tubular activity. The use of clearance methods necessitates the intravenous infusion of an osmotic diuretic (a salt, mannitol, glucose) and thereby counteracts the effect of the drug on water diuresis. This criticism also holds for the recent work of Craig, Visscher and Houck (15) in which they studied the effect of ether and cyclopropane on renal function. They found that in light anesthesia these anesthetics do not change tubular activity. However, since they infused large concentrations of glucose intravenously, the effect of these drugs on tubular function may have been masked by the concomitant glucose diuresis.

SUMMARY

1. Normal dogs, in the postabsorptive state and in water equilibrium, excreted ingested water (40 cc./kgm.) or intravenously infused water (25 cc./kgm) almost quantitatively within three hours

2. Phenobarbital sodium given intravenously in one-half of the anesthetic dose (0.04 gram/kgm), either 40 minutes after the administration of water by stomach or a few minutes before the intravenous infusion of water was started, inhibited water diuresis in every normal dog studied. The degree of this inhibition was such that the water output during the experimental period was about one-half of the water intake. Phenobarbital in doses smaller than 0.035–0 040 gram/kgm. had no consistent effect on water diuresis.

3. Phenobarbital sodium given intravenously in full anesthetic doses (0.08 gram/kgm.) inhibited water diuresis in normal dogs to a degree not more marked than that produced by the half anesthetic dose.

4. Still larger doses of phenobarbital sodium, causing very deep anesthesia, inhibited water diuresis to a greater degree in normal dogs.

5. In contrast to phenobarbital either amytal sodium or pentobarbital sodium, given intravenously in half anesthetic or even in full anesthetic doses, inhibited water excretion in only some animals. If an antidiuretic action was produced by either dose of these drugs, it was about of the same degree as that produced by the smallest effective dose of phenobarbital.

6. The effect of repeated administrations of pentobarbital sodium on the water diuresis of an individual normal dog was in some cases the same, but not always so; for instance, dogs which failed to respond with an antidiuretic effect on the first or first and second occasions gave an antidiuretic response at a later time, whereas sometimes the first and third administrations produced an antidiuretic response and the second failed to do so.

7. In dogs in which the entire neurohypophysis (infundibular process, infundibular stem and median eminence) was destroyed and in which, as a result, permanent diabetes insipidus developed, the excretion of water was never inhibited by any of the barbiturates studied even when these drugs were given in full anesthetic doses.

8. In contrast to the above, in dogs in which the usual hypophysectomy was performed, leaving some parts of the neurohypophysis intact, as evidenced by only a transient diabetes insipidus followed by a permanent normal water balance, the barbiturates inhibited water diuresis.

9. Phenobarbital, amytal, and pentobarbital sodium then, inhibited water diuresis in dogs only if some part of the neurohypophysis was functioning.

10. In contrast to its effect on water diuresis in normal dogs, phenobarbital, even in full anesthetic doses, either did not inhibit saline diuresis (isotonic sodium sulfate solution) or inhibited it only in some animals (isotonic sodium chloride solution).

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THE ABSORPTION OF THIOUREA FROM OINTMENTS APPLIED TO WOUNDS

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While early work indicated that thiourea was relatively nontoxic (1, 2), recent results in animals and in man demonstrate that thiourea and closely related compounds possess marked physiological activity. Upon absorption, these substances effect a reduction in the output of thyroid hormone and cause hyperplasia of the thyroid gland (3, 4). Both animal experiments (5, 6, 7) and clinical results of Astwood (8) and others have demonstrated that thiourea and the closely related thiouracil may produce blood dyscrasias affecting either the red or the white cells.

Results of these recent investigations raise a question as to the possibility of systemic effects following topical use of ointments containing thiourea as this material has been employed frequently as an antioxidant in preparations containing such substances as sulfonamides, tannic acid, and peroxides.

METHOD. Since histologic changes in the thyroid gland provide definite evidence of thiourea action, these were employed as a biological means of measuring absorption of thiourea from ointment bases. Additional observations were made on the blood of experimental animals, and wounded areas were examined for abnormal proliferation.

A total of 126 white rats weighing 160 to 220 grams, and equally distributed as to sex, were divided into ten groups. Circular wounds which included the skin and subcutaneous tissues and measured 1 cm. in diameter were prepared in the mid-dorsal anterior thoracic region of anesthetized animals These wounds were surrounded by cork rings which were held in place with Duo-Liquid Adhesive. Occasionally it was necessary to suture the rings to the skin.

Thiourea was incorporated in each of two different oil in water emulsion type bases. One of these contained tragacanth while the other did not. The concentrations of thiourea employed were 0 05, 0 1, 1.0, and 10 0 per cent. Two groups of animals served as controls and were treated with the ointment bases without thiourea. The other eight groups were treated with one or the other of the two bases containing one of the four concentrations of thiourea. Animals were housed in individual cages.

Approximately 100 mgm. of ointment was applied daily six days per week. Old ointment, eschars, and accumulated exudate were removed before each application. Hematologic examinations were made at the end of four weeks of treatment. Erythrocyte counts, leucocyte counts, and hemoglobin determinations were done on groups treated with 1 and 10 per cent thiourea and on controls. Differential cell counts were made on the animals treated with ointments containing 10 per cent thiourea.

At the end of two weeks' treatment, half of the animals in each group were sacrificed. Tissues from wounded areas and the thyroid glands were prepared for histological examination. The wounds of the remaining animals were re-established, the rings sutured in place, and treatment continued for two additional weeks. In the few instances when rings loosened, animals were discarded because of the possibility that they might have eaten some of the ointment.

An additional experiment was carried out to ascertain whether the thyroid glands of animals previously affected by thiourea would regress to give a normal histological picture. Twelve rats, half males and half females, were treated in the routine manner for 3 weeks with one of the ointments containing 10 per cent thiourea. At the end of this period treatment was discontinued, and four animals were sacrificed and their thyroids were examined microscopically. Four of the remaining animals were sacrificed at the end of 2 weeks and the balance at the end of 6 weeks. All thyroids were examined microscopically for evidence of regression.

RESULTS. The ointments containing less than 1 per cent thiourea had no significant effect on thyroid cells. Those containing 1 and 10 per cent thiourea caused various degrees of the typical change. This change consisted of an increase in height of the epithelial cells lining the follicles, a decrease and finally
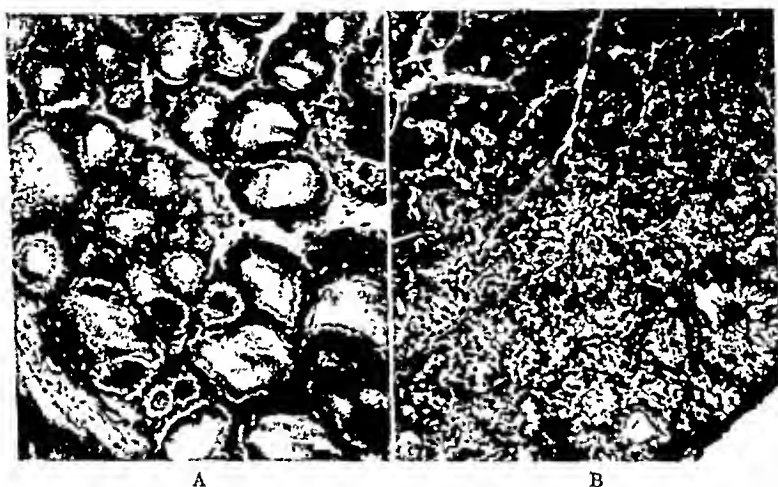
FIG. 1. "A" normal thyroid tissue from a control rat treated with an ointment containing no thiourea. "B" hyperplastic thyroid tissue from a rat treated with an ointment containing thiourea. This section shows the maximal effect observed. A description is given in the text.

a disappearance of colloid material, and a pronounced increase in the number of cells. A typical and maximal effect of this type is shown in figure 1. The ointment base containing tragacanth induced a greater histological response in all effective thiourea concentrations than did the other base. Differences in effects of two and four weeks' treatment and differences due to sex were not apparent.

Animals sacrificed two weeks after cessation of treatment showed no evidence of regression. However, the thyroids of those which were permitted to remain untreated for six weeks had either returned to normal or showed only a small degree of hyperplasia.

The examination of microscopic sections from treated areas revealed that the thiourea containing ointments caused no abnormal proliferation of tissue.

The hematologic studies indicate that none of the ointments definitely affected the blood picture. Average erythrocyte counts for the various groups ranged between 6.5 and 7.7 million cells per cu. mm. The hemoglobin concentration for animals in the group which was treated with 10 per cent thiourea in the tragacanth containing ointment was somewhat low and averaged 9.8 gm. per 100 cc. Hemoglobin concentrations for other groups, including controls, ranged between 11.0 and 11.7 gm. per 100 cc.

As might be expected, because of infections in the experimental wounds, leucocyte counts generally were high. They ranged for various groups between 25.0 and 35.5 thousand cells per cu. mm. Proportions of granulocytes and other white blood cells were within the normal range for rats. The average per cent of granulocytes for the two experimental groups was 22.7 and 23.4.

SUMMARY

Thiourea incorporated in, oil in water emulsion type, ointment bases in concentrations of 1 and 10 per cent was absorbed in sufficient amounts, when applied to wounds for periods of two weeks, to effect typical thyroid changes.

Lower concentrations of thiourea (0.05 and 0.1 per cent) did not cause thyroid hyperplasia when applied in a similar manner for periods as long as four weeks.

Absorption of thiourea was influenced by the vehicle as is evidenced by the finding that the tragacanth containing ointment produced the most marked effect.

Six weeks after discontinuing treatment, thyroid tissue had either returned to normal, or it showed only slight evidence of thiourea effect.

Thiourea applied in ointments caused no abnormal proliferation of tissue locally.

Under the conditions of these experiments thiourea had no effect on erythrocyte, leucocyte, and differential cell counts. Hemoglobin concentrations for one group of animals receiving thiourea were lower than for other groups. However, values for all groups, including controls, were low; and it is probable that the observed differences are not significant.

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ANTIBIOTIC SUBSTANCES ACTIVE AGAINST M. TUBERCULOSIS[1,2]

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Introduction. In 1912 Vaudremer observed that a strain of *Aspergillus fumigatus* produced (in the mycelium) a substance which neutralized tuberculin (1) and was capable *in vitro* of destroying the pathogenicity of *M. tuberculosis* (2). Recently Soltys (3), and Asheshov and Strelitz (4) reported that culture fluids from strains of *A. fumigatus*, which were isolated independently, exhibited inhibitory action against *M. tuberculosis in vitro*. It is not yet reported whether these effects can be attributed to one or more of the four antibiotic substances which have been obtained in pure form from various strains of *A. fumigatus* [summarized by Waksman and Geiger (5)]. Schatz and Waksman (23) have shown that fumigacin inhibits M. tuberculosis.

A strain of mold believed to be a *Penicillium* has been reported to cause reduction in the virulence of *M. tuberculosis* (6). Waksman and Woodruff (7) have found an antibiotic substance, streptothricin, obtained from cultures of *Actinomyces lavendulae*, to be active *in vitro* against *M. phlei;* and Schatz, Bugie and Waksman (8) have described the closely related streptomycin, from *Actinomyces griseus*, which inhibits both *M. phlei* and *M. tuberculosis*. Preliminary studies, in which streptomycin gave some protection to guinea pigs against *M. tuberculosis*, have been described by Feldman and Hinshaw (9).

About a year ago a species of *Aspergillus* was isolated by one of us (R. C. A.), who found that when grown on certain media it gave a culture fluid which inhibited *M. tuberculosis in vitro*. Some time later we undertook the study of the production and isolation of the antibiotic material. A pure line strain of the mold, obtained by single spore cultivation (10), was kindly identified by Dr. Kenneth B. Raper[3] as a strain of *Aspergillus flavus* Link. Early in our work (before Dr. Raper's study was made) with this strain we became aware that ours was very closely related to or identical with the "aspergillic acid problem".

Antibiotic activity was first detected in cultures of an *A. flavus* by White (11) and impure aspergillic acid was obtained from the cultures by White and Hill (12). It has been studied in greater detail by Jones, Rake and Hamre (13). Pure aspergillic acid (m.p. 93°) was first reported by Menzel, Wintersteiner and Rake (14) and more recently its chemical nature has been elucidated by Dutcher and

Wintersteiner (15). None of these investigators has reported what amount of the pure fraction of m.p. 93° was obtained from the crude antibiotic material. High melting fractions are mentioned by White and Hill, and by Menzel, Wintersteiner and Rake who state that a compound, m.p. 149°, having $\frac{1}{10}$ the antibiotic activity of pure aspergillic acid, was obtained. Dutcher (private communication) reports that an *A. flavus* produces a mixture of aspergillic acid and hydroxyaspergillic acid (m.p. 120°), the crude mixture melting between 97° and 107°. The hydroxyaspergillic acid "has an extremely low antibiotic activity in comparison with aspergillic acid".

Although our work is by no means completed, a description of our progress might be of value, particularly in view of the demonstration of the activity of aspergillic acid like substances on *M. tuberculosis* (Bush, Dickison, Ward and Avery)[2].

EXPERIMENTAL. Culturing of the mold has been carried out solely in shallow, quiet media of various compositions. Of the latter we have found none equal to lactose and Difco peptone in distilled water, and the minimum concentrations of these solutes which produce maximum yields seem to be 2% of each. The medium was distributed in one or two liter batches in large diphtheria toxin bottles and sterilized in the autoclave. Inoculation was performed by pouring in 1–2 cc. of a thoroughly agitated, freshly sporulated culture of the mold. After growing for 5–6 days at 30–35°C. the one-liter (1–1.5 cm. deep) cultures had developed maximum activity and the two-liter (2.5–3 cm. deep) batches were about half as active. After a total of 12–14 days the deeper cultures reached the maximum activity.

Assays were carried out against *Staphylococcus aureus* and *Escherichia coli* by a serial dilution method[4] (16) and against *M. tuberculosis* by an adaptation of the plate-cup method[5] commonly used for assay of penicillin (17, 18). We have taken as one *M. tuberculosis* unit (TBU) the amount of antibiotic dissolved in 1 cc. which gives a 20 mm. diameter clear zone under standard conditions.[5] At maximum titer the cultures contain 1–2 TBU per cc. and 20–40 dilution units per cc against both *S. aureus* (SU) and *E. coli* (E. coli U.). This titer remains constant for months.

Extraction of the antibiotic material from the culture fluid at pH 2–3 has been carried out by means of the spray extraction column described elsewhere (16, 19, 20). Isopropyl acetate or benzene or heptane (Eastman Practical) are satisfactory solvents.

[4] Sterile filtrates can be made through either Seitz or glass bacteria filters; no difference in titer could be detected. The former filters cause precipitation in filtrates, particularly if the concentration is greater than 1 mg /cc. Filtrates made through glass remain perfectly clear.

[5] A loopful of a 24–48 hour pellicle of a quick-growing strain of *M. tuberculosis* (R. C. A.) grown on Difco nutrient broth, is placed on the nutrient agar, and smeared almost uniformly over the surface with a spatula The "penicylinders" are heated and placed on the agar. Of the solution to be tested 0.2 cc. is pipetted into the cup. The dish is covered with a porous top and incubated at 35°C. The diameters of the clear zones are measured after 24 and after 48 hours. The two measurements are usually nearly identical. Control tests with aqueous 0.05 N NaOH sooution give no inhibition, but 0.1 N sometimes gives a small clear zone. Most test solutions are therefore diluted to 0.05 N or lower. The error of our plate assay values is about ±15%.

It is important that after the agar is poured (25 cc. Difco Nutrient Agar, 1.5%, in 20 × 100 mm. Petri dishes) the plates be kept perfectly level in order to obtain a uniform depth (18). The size of the clear zone is inversely proportional to this depth. Petri dishes with perfectly flat bottoms should give more uniform results.

Early extractions were carried out with isopropyl acetate and it was found that the antibiotic material was removed from this solvent only in very low yield by aqueous NaHCO₃. Even aqueous NaOH at concentrations sufficient to give pH 11–12 did not extract good yields. Distillation of this solvent and treatment of the residue with benzene and excess (pH 9–10) aqueous NaOH (0.1–0.5 N) was found to give fair yields in the aqueous alkaline solution.

Later extractions of the culture fluid at pH 2–3 were carried out with benzene in the spray column. It was found necessary to spray the culture through the column a second time to get nearly maximum recoveries of the extractable materials. If heptane were used as extracting solvent three passes were necessary, but the activity of the product was somewhat higher. The following is a description of a typical experiment:

The culture fluid was poured from the culture bottles through glass wool. This pooled culture (71.4 liters, assay: 2 TBU, 23 SU and 23 E. coli U. per cc.) was acidified to pH 2.6 with concentrated HCl and sprayed through the column of heptane at an average rate of 12–15 liters per hour, while fresh heptane was run through counter-current-wise at 2 liters per hour. The heptane extract (10.7 liters) was distilled to a volume 212 cc. and reserved as "heptane 1". The acid culture fluid was put through this extraction procedure a second and a third time, and the concentrated extracts "heptane 2" (149 cc.) and "heptane 3" (97 cc.) obtained. By evaporation of small portions to dryness and weighing the crystalline residues, these extracts were found to contain, respectively, 13.9, 7.3 and 3.0 grams of relatively non-volatile solute. The activity of all three fractions was 3.0 TBU per milligram; this represented a total of 73,000 TBU, about 50–75% of the apparent activity of the culture fluid.

Preliminary purification of this crude material was carried out as follows: The main portions of the three heptanes (representing 99% of the total) were combined in a separatory funnel with 400 cc. of distilled water and the active material was taken into the aqueous phase by adding 10 cc. portions of N NaOH and equilibrating by shaking 2 minutes each time. A total of 80 cc. of the alkali gave pH 7.95, and two more portions of 5 cc. each gave pH 8.65 (the break in the titration curve occurred at pH 8.3). The aqueous phase was drawn off (small amounts of emulsion were centrifuged), and the heptane layer was equilibrated with 200 cc. of 0.025 N NaOH. This aqueous layer was separated (pH 11.7). The heptane layer was rich orange in color. The two aqueous alkaline solutions were extracted in order and in succession with a fresh portion of heptane (200 cc.) which raised the pH of the first to 8.95, and lowered the pH of the second to 11.5. This second heptane was yellow. The two aqueous solutions had volumes 500 cc. and 195 cc., and showed on assay, respectively, 160 and 18 TBU per cc. The first solution gave assay values of 2200 E. coli U. and 2200 SU per cc. The yield from the culture at this stage was 70 ±15% for all three types of units.

These two aqueous solutions were combined, acidified to pH 1.2 with 10 cc. of concentrated HCl and shaken 2 minutes with 500 cc. of heptane. The aqueous solution was drawn off and extracted with 200 cc. of fresh heptane. The aqueous phase retained much color. The two heptane extracts (515 cc. and 197 cc.) contained, respectively, 36.5 mg./cc. and 2.62 mg./cc. of crystalline relatively non-volatile solute. They were combined, mixed well, stored in a glass stoppered pyrex bottle at room temperature, labelled "crude acids in heptane" and used in the following experiments.

Properties of the "crude acids in heptane". When 5.0 cc. of this solution were evaporated (quickly, to minimize loss of solute) the oily residue (136.6 mg.) soon became completely crystalline. It melted 82–92°, and gave [α]_D + 9.7 (c = 4 in absolute methanol) and [α]_D + 10.8 (c = 4 in 95% ethanol). It was dissolved by the following solvents in order of increasing amounts (mg./cc. at 20–25°): water (0.5–1), heptane (50), acetone (300), methyl alcohol benzene, isopropyl acetate.

Extraction of 5.00 cc. with 5.00 cc. concentrated CP HCl, separation and evaporation of the heptane layer left 1.4 mg. (1%).

Extraction of 5.00 cc. with 10.00 cc. of 0.07 N NaOH, separation and evaporation of the

heptane layer left 1.5 mg. Assay of the alkaline aqueous extract showed 4 TBU, 70 E. coli U. and 70 SU per mg. of solute calculated by difference as free acid(s). (Yield from culture: 80 ± 15%.) A 1.00 cc. portion of this alkaline aqueous solution was diluted with 2.0 cc. of 0.1 N NaOH, the test tube corked tightly and heated in a bath of boiling water for 30 minutes, cooled, and the contents diluted with distilled water for assays. The activity against the three micro-organisms was apparently not different from that of the unheated solution.

TABLE 1

Antibiotic activity of "crude acids in heptane"

	DILUTION UNITS PER MG.
In Difco nutrient broth	
Ps. aeruginosa..	<1†
Pr. vulgaris B*...	5
Past. pestis...	15
Kleb. pneumoniae...	20
Pr. vulgaris H*..	20
B. aerogenes D*..	40
B. proteus OX 19...	50
B. anthracis...	50
V. cholerae..	55
E. typhosa*..	60
St. aureus...	70
St. albus..	80
S. paratyphi A...	80
B. subtilis..	90
S. paratyphi B...	90
C. diphtheriae gravis..	100
S. dysenteriae...	150
Br. suis...	160
Str. hemolyticus...	170
S. paradysenteriae...	600
In Difco nutrient broth + 2% blood	
Str. viridans..	10
Pneumococcus Type 1..	<5
St. aureus...	25

* These organisms were isolated from patients at Vanderbilt University Hospital.

† Considerable inhibition of growth at 1 mg./cc.

This aqueous solution of the salts of the crude acids has been tested against bacteria in Difco nutrient broth. The results are shown in table 1. When similar dilutions were made in spinal fluid (dog) as a culture medium the activity against *S. aureus* and *E. coli* was the same as in nutrient broth. When plasma, laked defibrinated blood, or serum (rabbit) was used, the activity against these organisms was greatly reduced.

A solution of the crude acids containing 0.25 mg./cc. as sodium salts gave our standard zone of inhibition[5] against *M. tuberculosis* (R. C. A.) and the activity was of the same order of magnitude against the following acid-fast organisms (American Type Culture Collection numbers in brackets): *M. phlei* (355), *M. leprae* (4243), *M. smegmatis* (101), *M. chelonei* (114), *M. berolinensis* (20), and *M. arium* (7992). All showed up well within

48 hours on nutrient agar $+ 2\%$ glycerine. Similar activity was shown against a species of monilia.

Purification of the crude acids has been carried out with partial success by fractional crystallizations and by fractional extractions, and by a combination of these methods. Many experiments indicate that the mixture contains two or more very closely related substances which appear to have very similar antibiotic properties.

Fractional crystallizations were carried out with the aid of centrifugal filters (figure 1). When 6.4 grams of the crude was recrystallized from 5.0 cc. of acetone, 4.8 grams, m.p.
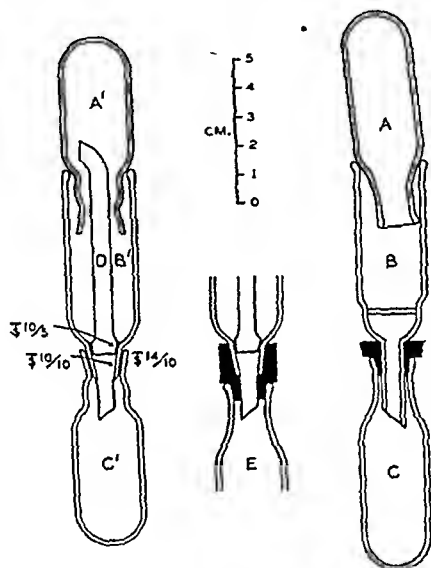
FIG. 1. CENTRIFUGAL FILTERS

These filtering assemblies are somewhat different from those described by Moore and Stein (21) and by Craig and Post (22). They have these advantages: the bottles A and C (A' and C') are interchangeable; the male grinding D is of standard taper; several "porosities" can be had with the same filter body B' by regrinding commercial ST joints on a cast iron standard taper tool with carborundum of desired sizes—say 150 to 100 mesh. The lengths of the grindings D can be adjusted by cutting off and grinding down the commercial joints as far as desired. A 5 mm. length ground with 150 mesh carborundum gives a filtration rate of 1-2 cc. per minute of distilled water at about 1000 r.p.m. The assemblies fit in the 100 cc. brass cups in the No. 2 International Centrifuge. B is a Corning F filter. For speeds above 1000 r.p.m. a rubber collar may be put between A and B. A'B'C'D may not withstand speeds much greater than this. A tiny vent should be punched in the neck of each of the bottles A and C.

93-95° was obtained; two recrystallizations in succession from 3 cc. portions of methanol gave 2.9 grams, m.p. 96.5-98°; from 2.5 cc. acetone this yielded 2.5 grams of light yellow crystals, m.p. 97.5-98°. This had $[\alpha]_D + 10.8$ (c = 4 in 95% ethanol); %N 10.76 (micro Kjeldahl). It had 100 ± 15 SU and E. coli U. per mg. This fraction was not pure because further recrystallizations resulted in a gradual rise of the melting point. Systematic recrystallization of the crude has led to a small amount of material of m.p. 118-121°. Most of the intermediate fractions comprising about half the total weight of the crude, taken at the start, have m.p. between 98° and 108°.

The fraction of m.p. 118-121° was titrated electrometrically with aqueous NaOH and found to have a neutralization equivalent of 230 and apparent ionization constant of pK' 5.5.

This high melting fraction has not yet been rigorously tested for homogeneity. Its properties indicate that it could be (largely) an isomer of the fraction of m.p. 96.5–97.5 to be described later in this paper.

Systematic multiple fractional extractions (16). The first experiments in which this method was used were made with crude acids extracted from the culture fluid with benzene, and partly purified as described for the heptane-extracted material leading to the "crude acids in heptane". A solution containing 3000 mg. of solute in 105 cc. of heptane (activity per mg.: 4 TBU, 80 SU, 80 E. coli U.) was extracted in succession with six 2000 cc. portions of 2% aqueous NaCl solution. The aqueous salt solutions were then extracted in order and in succession with three 100 cc. portions of fresh heptane. The distributions of mg. of solute and of units per mg. against *M. tuberculosis*, *S. aureus* and *E. coli* were:

> Heptane I₆, 375, 4, 70, 70
> Heptane II₆, 545, 5, 90, 90.
> Heptane III₆, 404, 4.5, 90, 90
> Heptane IV₆, 290, 4, 90, 90

Most water fractions contained very little or no activity, but the fraction "W IV⁴" was unique, having 12 SU and 12 E. coli U. and about 0.5 TBU per cc. When this water solution was extracted with 1 liter of heptane, and this distilled to small volume, there was obtained 300 mg. of crystalline material. At the end of the distillation the residue was inadvertently superheated and assays showed only 2.0 TBU/mg. remaining—a loss of about 50% from the activity of "W IV₄"⁶. The only great difference between the heptane fractions was in the appearance of the crystalline residues: H I₆ was rich yellow and oily; H II₆ was light yellow and "waxy"; H III₆ was very light yellow and beautifully crystalline; H IV₄ was yellow and crystalline but somewhat "waxy".

The fraction H III₄ was recrystallized as follows: Some 83% of the heptane solution (containing 333 mg. of solute) was distilled to a volume of 5.0 cc. and transferred quantitatively to a centrifugal filtration tube with the aid of 1.0 cc. of fresh heptane. After seeding and standing overnight at 5° the crystals were filtered in the centrifuge and allowed to dry to constant weight in air; net: 222 mg , m.p. 95–6°. Recrystallization from 1.1 cc. of absolute methanol at 5° and quick washing in the centrifugal filter with 0.5 cc. cold CH_3OH gave 103 mg. bright yellow, massive prisms, m.p. 96.3–97.3°. A second crop of about 20 mg. was combined with this and recrystallized again from 0.8 cc. of methanol, yield 98 mg., m.p. 96.6–97.7°. This was recrystallized from 2 cc. of redistilled heptane to give 95 mg., m.p. 96.5–97.5°. This apparently pure substance was compared with a sample of aspergillic acid kindly supplied by Dr. J. D. Dutcher of the Squibb Institute for Medical Research. Under our conditions⁷ the aspergillic acid had m.p. 93.5–96.0 (softens 92.5) and a finely ground, approximately 1:1 mixture with the compound of m.p. 96.5–97.5° melted 94.5–96.5°.

When the substance of m.p. 96 5–97.5° was titrated electrometrically with alkali the neutralization equivalent was 230 (9.88 mg. in 0.5 cc. CH_3OH + 10 cc. distilled water, boiled and cooled, required 0.850 cc. of 0.0502 N NaOH) and the point of half neutralization gave pK' 5.5 (ca. 25°). Both of these values are in close agreement with those published for aspergillic acid (Dutcher and Wintersteiner).

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⁶ This "water-soluble", relatively unstable fraction was not found in the heptane-extracted material fractionated later. Possibly distillation of the heptane extract (90–100°C.) caused greater loss of relatively unstable fractions than distillation of the benzene extract (80°C.). Concentration of the crude acids by extraction will be investigated, in order to recover the unstable active substance(s). Solubility studies on crystalline material, made after this manuscript was submitted have shown that treatment with boiling heptane causes an increase in solubility!

⁷ All melting points reported in this paper are °C. corr., and were taken in open capillary tubes (the lime glass tubing from which these were drawn was acid-washed) in an electrically heated, well stirred oil bath, rate of heating 1.0 to 0.5° per minute.

Aspergillic acid (Dutcher) was found to have antibiotic activity of 4 ± 1 TBU, 70 ± 10 SU and 70 ± 10 E. coli U. per mg. The substance of m.p. 96.5–97.5° had 4 ± 1 TBU, 100 ± 10 SU and 100 ± 10 E. coli U. per mg. The addition of 0.1 cc. of defibrinated blood (rabbit) to each 5 cc. portion of broth and retesting resulted in a greatly reduced activity (about 75%) for both substances.

We are not yet sure whether the small differences between these two specimens should be attributed to slight differences in structure or to a difference in purity. It is perhaps important to note that melting points of mixtures of aspergillic acid (Dutcher) and our higher melting fractions (for example, 109–111° and 112–115°) always lie between the melting points of the components.

The material of m.p. 96.5–97.5 will be more rigorously tested for homogeneity.

The preceding systematic multiple fractional extraction procedure has been carried to a more advanced stage with a 230 cc. portion of the "crude acids in heptane" (27.3 mg./cc.) which was concentrated first to a volume of 105 cc. This solution (60 mg./cc.) was extracted in succession with twelve 2000 cc. portions of 2% salt solution. The aqueous solutions were extracted in order and in succession with eleven 100 cc. portions of fresh heptane. (All the water fractions had pH values 4.2–4.6). Assays showed that all water fractions had practically no antibiotic activity. The distribution of solute and of antibiotic activity in the twelve heptanes is shown in figure 2.

In order to demonstrate whether the small differences shown in the SU/mg. and E. coli U./mg. were significant, the final assays were carried out so that ten tubes were used at each of two dilutions, 10–15% above and 10–15% below the approximate median growth point. In these groups of 10, there were always 8–10 tubes free of visible growth at the lower dilution and 8–10 tubes showing unmistakeable growth at the higher dilution. The error of the estimated median end-point is thus not greater than about ±10%. (All of our dilution assays (16) are estimates of the point at which half the tubes show growth in 24 hours and could well be expressed as SU/50.)

The fractions H III$_{12}$ − H VIII$_{12}$ inclusive represent antibiotic material which could be very largely one substance. It seems likely that this will turn out to be the substance described above, of m.p. 96.5–97.5°. Application of crystallization procedures is being made to isolate this substance, and its homogeneity will be tested by further application of the fractional extraction method.

The fractions H I$_{12}$, H II$_{12}$, and particularly H IX$_{12}$ − H XII$_{12}$, show activity slightly different from the intermediate fractions. It was found that some six weeks after completion of a first series of assays, H I$_{12}$ had lost over 50% and H II$_{12}$ over 25% of the earlier activity while all other fractions had remained constant (all were stored at room temperature in pyrex glass stoppered bottles—stoppers wet with glycerine to prevent evaporation). The curves suggest that in the crude at least two, possibly three, substances are responsible in different degree for the antibiotic activity against S. aureus and E. coli.

Thus the fractionation apparently separated (in H I$_{12}$) a less stable antibiotic substance different from the material in H III$_{12}$ and subsequent fractions and

also different from the fraction W IV$_4$ obtained in the previously described fractionation. It is, however, just possible that some other substance in H I$_{12}$ and H II$_{12}$ caused the instability of an antibiotic substance identical with that in other fractions. For example, H I$_{12}$ was quite yellow, H II$_{12}$ moderately yellow, the others only faintly yellow.

The amounts of H I$_{12}$ and H II$_{12}$ are such that had the original crude mixture been very carefully tested for loss of activity on standing, the assays could not have detected the loss.
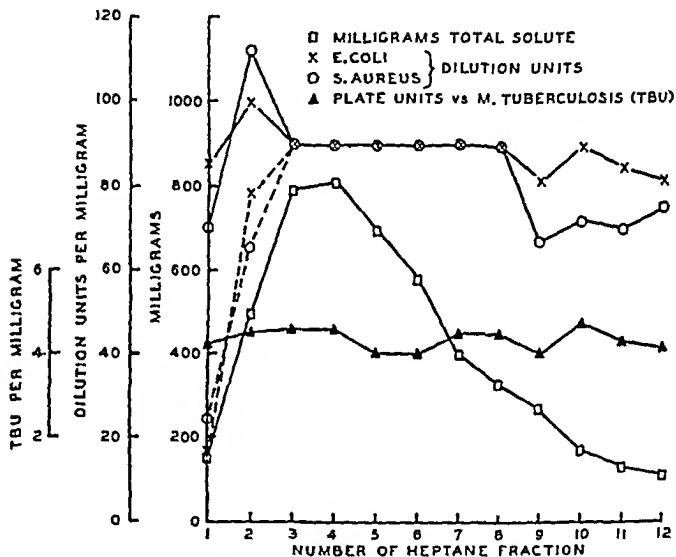
FIG. 2. FRACTIONATION BY MULTIPLE EXTRACTION

These curves represent the distribution in the twelve heptane fractions (see experimental) of the partially purified material called "crude acids in heptane", after the performance of systematic multiple fractional extractions with aqueous salt solutions. The numbers of the heptane fractions 1, 2, and so forth correspond to the symbols I$_{12}$, II$_{12}$, and so forth, used in the text (where the subscripts denote the number of extractions through which the solution has passed).

The dotted lines show the fall in activity after the solutions stood at room temperature in diffuse daylight for about six weeks.

It is hoped that further investigation of these fractionation procedures will lead to the isolation in pure form of the several fractions indicated to be present.

Studies in mice. The crude acids were extracted from the heptane with a slight excess of 0.1 N NaOH. When this aqueous solution (10 mg. crude acids/cc.) was injected intraperitoneally into mice, no symptoms were produced by a dose of 50 mg./kg.; 100 mg./kg. killed most of the mice within an hour, with convulsions and symptoms of respiratory paralysis. (These doses and symptoms are comparable to the data given by White and Hill). To test the rate at which the drug(s) were inactivated or excreted, the dose of 50 mg./kg.

was given repetitively: eight such doses repeated at 1 hour intervals produced no symptoms in any of 10 mice; when repeated at $\frac{1}{2}$ hour intervals a similar result was obtained; at 15 minute intervals 3 mice were killed by, and 4 survived, 8 doses.

These results indicate that if the antibiotic substances are active *in vivo*, administration will have to be frequent or continuous in order to protect the animals from an experimental infection. (It is possible, however, that the toxicity and antibiotic activity are due in different degree to different components of this impure material.)

Studies in dogs. In order to prepare an approximately isotonic solution at pH 7.4, the "crude acids in heptane" were extracted with 0.66 the equivalent amount of 0.15 N NaOH. This aqueous solution contained 33 mg. crude acids per cc. Careful assays showed no significant difference from the antibiotic activity obtained when the pH of the aqueous extract was 10–11.

A dog (15 kg.) was given 10 mg./kg. intravenously during half a minute. As no symptoms were seen an additional 20 mg./kg. were given $\frac{1}{4}$ hour later, injected during 45 seconds. Respiration was stopped and the animal was anesthetized, but after a minute breathing commenced and reflexes began to return, and within 3–5 minutes the animal appeared normal. No further symptoms were observed. Samples of blood and spinal fluid were taken 15 minutes after the injection; assays showed no antibiotic activity in either; that is, less than 0.3 TBU/cc. The dog was kept in a metabolism cage for collection of urine. For over 48 hours no urine was passed. Shortly after this time 1710 cc. was voided. Direct assay of a sample of this showed very little antibiotic activity. The whole batch of urine was acidified to pH 3.2 and extracted with 200 cc. of heptane. Evaporation of 5 cc. of this extract left 1.4 mg. of relatively nonvolatile residue, a yellow oily material. Extraction of 5 cc. of the heptane solution with 5 cc. of .05 N NaOH left in the heptane 1.1 mg. of solute. The aqueous alkaline solution (total organic solute about 0.3 mg.) contained 25 E. coli U. and 50 SU. This represented in the total extract of the urine the equivalent of roughly 0.3% of the antibiotic injected into the dog.

The isotonic solution of pH 7.4 was administered intracisternally to several dogs[*]. A dose of 33 mg. (1 cc.) in a dog of 8 kg. caused convulsions and death within 20 minutes. Sixteen mg. in another animal of the same size caused no symptoms. The cell count of the spinal fluid was raised only moderately during the next few hours. A sample of spinal fluid taken 30 minutes after the injection (from the site of the injection) showed an activity of 1 TBU/cc. After an hour no activity was detected in the spinal fluid.

SUMMARY

A mold identified as a strain of *Aspergillus flavus* Link has been isolated and when grown on a peptone-lactose medium has been found to produce a culture fluid which inhibits *M. tuberculosis*, *S. aureus*, and *E. coli in vitro*.

Antibiotic material has been extracted from this culture fluid at low pH by organic solvents (benzene or heptane).

This extract yields a mixture of organic acids which has antibiotic activity *in vitro* against a wide variety of both gram negative and gram positive bacteria, and against a number of acid-fast organisms. Activity is also shown against a strain of *monilia*. The properties of the crude antibiotic material are very similar to those described for aspergillic acid.

Fractionation procedures involving multiple extractions and recrystallizations have led to the isolation of an apparently pure aspergillic acid-like substance, m.p. 96.5–97.5° corr., in 5% yield, and a fraction m.p. 118–121° having about 75% the antibiotic activity, the same neutralization equivalent (230) and pK' (5.5) as the substance of m.p. 96.5–97.5.

There is good evidence that *at least* one other somewhat less stable antibiotic substance is present in the crude mixture.

All these fractions seem to have a similar antibiotic activity against *S. aureus*, *E. coli* and *M. tuberculosis*, and the addition of blood to the medium greatly reduces this activity. The activity is not reduced by spinal fluid.

Studies on impure material have shown that the toxic effects and the antibiotic activity disappear rapidly after systemic or intrathecal administration (mice, dogs) and that antibiotic material is excreted in urine (dog) in very small amount.

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SULFONAMIDES FOR BACILLARY DYSENTERY

I. The Antibacterial Activity of Sulfacarboxythiazoles and Sulfathiadiazole

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Since a rational approach to the chemotherapeutic control of intestinal infections was established through the introduction of sulfaguanidine by Marshall in 1940 (1), this drug and others with similar properties have been used with considerable success in the treatment of bacillary dysentery.

The basic idea at present is to use active drugs, highly soluble at intestinal pH's, but absorbed and excreted in such a manner as to maintain high concentrations in the intestinal contents and low concentrations in the blood. If the blood level remains low because of a very rapid rate of excretion, then, in order to avoid kidney complications, a high urine solubility is also necessary.

In a search for new chemotherapeutic drugs which may have more of the desirable properties outlined above, we have turned our attention to some of the more acidic sulfonamides. Increased acidity greatly increases the limiting solubilities of these compounds in the intestinal and urinary tracts. Furthermore, oral dosage with this type of compound does not result in high blood levels. This may possibly be due either to low permeability of the intestinal mucosa to the ionic form of the drugs, or to a rapid rate of excretion in the urine, or both (2).

In this connection we have investigated 2-sulfanilamido-1,3,4-thiadiazole (sulfathiadiazole), 2-sulfanilamido-5-carboxythiazole (sulfacarboxythiazole), 2-sulfanilamido-4-methyl-5-carboxythiazole and 2-sulfanilamido-4-carboxythiazole. Data will be presented to show that sulfacarboxythiazole and 2-sulfanilamido-4-methyl-5-carboxythiazole owe their activities to decarboxylation to sulfathiazole and to 2-sulfanilamido-4-methylthiazole, respectively. Because of the ease with which decarboxylation of the methyl compound occurred and because of the possibility of toxic reactions from the sulfamethylthiazole derived from it (3), this compound was not considered further. The 2-sulfanilamido-4-carboxythiazole did not decarboxylate and had only a slight degree of antibacterial activity. Of the four compounds mentioned above, only sulfathiadiazole (which appeared to be chemically stable) and sulfacarboxythiazole (which appeared to decompose to sulfathiazole) had characteristics warranting further study with respect to possible usefulness in the treatment of intestinal infections.

The synthesis and physical properties of sulfathiadiazole (4, 5) 2-sulfanilamido-4-methyl-5-carboxythiazole (6) and 2-sulfanilamido-4-carboxythiazole (7) have been reported. Sulfacarboxythiazole was synthesized in this laboratory by H.

W. Marson. The material melted with decomposition at 209–210°C. This decomposition point depends somewhat on the rate of heating. The *in vitro* activity of sulfathiadiazole has been reported to be about equal to that of sulfanilamide (4, 5, 8). A recent preliminary clinical study has indicated that sulfacarboxythiazole may be of use in the treatment of enteric infections (9).

The results of a study of the comparative antibacterial activity of sulfathiadiazole and sulfacarboxythiazole, together with sulfathiazole, sulfadiazine, sulfaguanidine and sulfathalidine (N⁴-phthalysulfathiazole) are given in the present paper. Experimental studies on the absorption, excretion, conjugation and toxicity of sulfathiadiazole and sulfacarboxythiazole will be reported elsewhere.

EXPERIMENTAL. *Buffer solubilities:* Buffers consisting of 0.05 M phosphate plus 0.025 M citrate and covering the physiological range of urinary and intestinal pH, were saturated with the compound in question by stirring 24 hours at 37°C. The excess solid was filtered off at 37°C.; final pH was measured; and determinations of the dissolved compound were made by the Bratton and Marshall method (10). The results obtained with some of the sulfonamides which have been used in bacillary dysentery, along with sulfacarboxythiazole and sulfathiadiazole, are given in fig. 1. The sulfadiazine values are those of Gilligan and Plummer (11). With respect to fig. 1, it should be remembered that this solubility scale cannot be extrapolated due to the fact that it is logarithmic. The curves as drawn cover the pH range actually measured. The results for sulfacarboxythiazole include any sulfathiazole which might have been formed during the 24 hour stirring period. However, as will be shown in the next section, this would be only a very small amount of sulfathiazole.

Chemical stability of the carboxythiazoles: Early in the course of investigating the carboxythiazoles, several observations seemed to indicate that sulfacarboxythiazole and 2-sulfanilamido-4-methyl-5-carboxythiazole were undergoing decomposition. When solutions of these compounds were autoclaved, a decrease in solubility occurred. Also, titration with alkali and acid indicated that a change was taking place in solution which increased titratable acid in the carbonate pH range.

In order to ascertain whether sterilization of bacteriostatic test solutions by autoclaving resulted in decomposition of the compounds, the following comparison was made. A portion of a solution sterilized by Seitz filtration was autoclaved and then titrated for bacteriostatic activity along with the original filtered solution. The results given in table 1 clearly indicate that autoclaving increased the activity of solutions of the two above-mentioned compounds.

In another experiment, more concentrated solutions of the three carboxythiazoles, prepared in 1.0 M phosphate buffer at pH 7.0, were autoclaved and incubated at 37°C. for 72 hours. During this period crystals separated from the sulfacarboxythiazole and 2-sulfanilamido-4-methyl-5-carboxythiazole solutions. These solids were identified by melting points, mixed melting points, chemical analysis and ultraviolet spectral curves, as sulfathiazole and sulfamethylthiazole, respectively. There was no evidence of any decarboxylation of 2-sulfanilamido-4-carboxythiazole under these conditions. With respect to the methyl com-
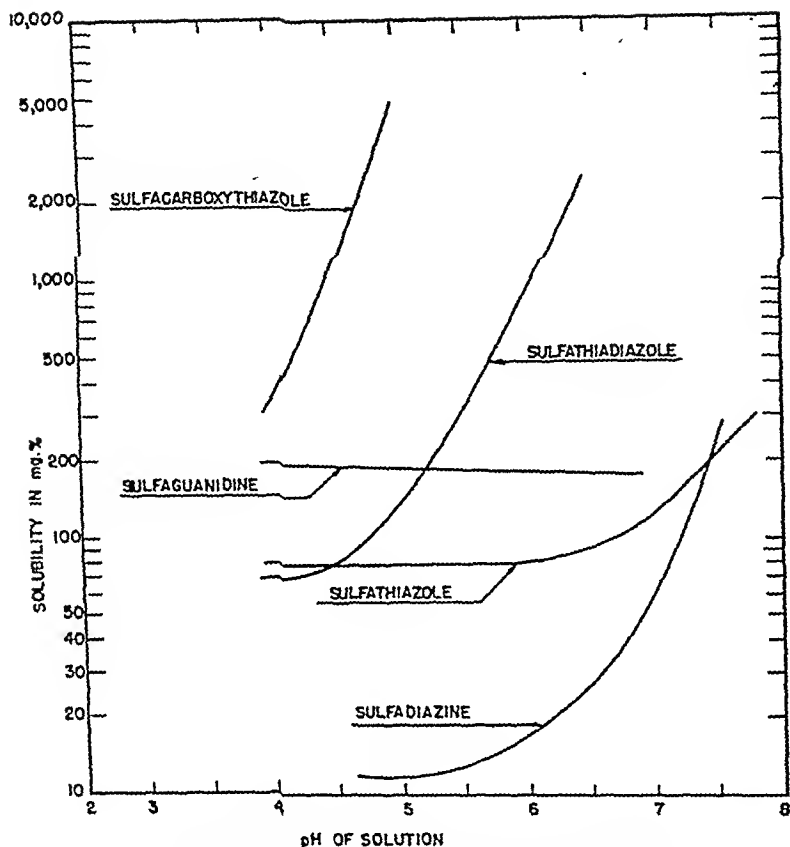
FIG. 1. Solubilities of Sulfanilamides in Buffers

Phosphate (0 05 M) and citrate (0 025 M) at 37°C. pH values are those of saturated solutions. Data for sulfadiazine (Gilligan and Plummer) in 0.066 M phosphate (11).

TABLE 1

The effect of autoclaving on the bacteriostatic activities of solutions of the sulfathiazoles

COMPOUND	BACTERIOSTATIC CONC. IN MGM. %			
	Autoclaved		Filtered	
	24 hrs	48 hrs	24 hrs	48 hrs.
2-S-Thiazole (Sulfathiazole)	1/64	1/32	1/64	1/32
2-S-5-Carboxythiazole (Sulfacarboxythiazole)	1/8	1/4	1	2
2 S-4 Methylthiazole	1/32	1/16		
2-S-4 Methyl-5-carboxythiazole	1/16	1/8	1	1
2-S-4-Carboxythiazole	4	8		

S = sulfanilamido group.

Test medium McLeod's synthetic (12) buffered at pH 7.2.

Inoculum *E. coli*, about 500 bacteria per ml.

Incubation at 37°C

pound, our results confirm those of Jensen and Thorsteinsson (6).   These qualitative results indicated that possibly all of the activity of the carboxythiazoles tested in table 1 might be due to the corresponding sulfathiazole being present as an impurity, or being released during the experiment.

Quantitative estimates of the amount of this breakdown were based upon ultraviolet absorption curves obtained with a Beckman spectrophotometer using 3.74×10^{-5} M solutions of the compounds in 0.05 M phosphate buffer at pH 7.0. The results obtained before and after autoclaving and incubating are given in
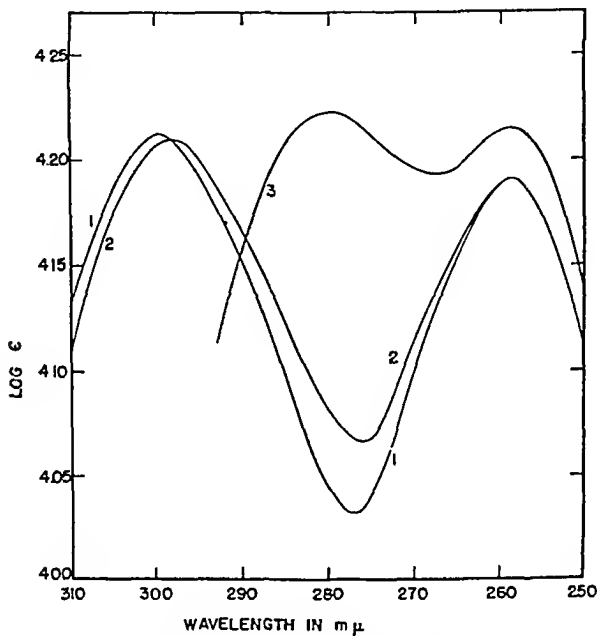
Fig. 2. Ultraviolet Absorption Curves of Sulfacarboxythiazole and Sulfathiazole

3.74×10^{-4} M solutions in 0.05 M phosphate buffer at pH = 7.0   Curve 1, sulfacarboxythiazole before autoclaving; Curve 2, solution of Curve 1 after autoclaving and 72-hour incubation at 37°C.; Curve 3, sulfathiazole before and after similar autoclaving and incubation treatment.

figs. 2 and 3.   From a study of mixtures of the proper compounds it was possible to show that the position of the peak, associated with the thiazole portion of the molecule, (wavelength 300-280 mμ), could be related quantitatively with the percentages of the compounds in the solution being measured.   On the basis of such calibration curves, it was estimated that $12.0 \pm 2.5\%$ of the sulfacarboxythiazole decomposed to sulfathiazole and that 100% decarboxylation of the 2-sulfanilamido-4-methyl-5-carboxythiazole occurred.   By use of the same method of analysis, the rates of breakdown of the two compounds, at pH 7.0 and 37°C.,

were determined. Both appeared to decompose according to a first order rate law:

$$k = \frac{2.303}{t} \log \frac{C_0}{C}, \quad \text{where} \quad \begin{aligned} k &= \textit{reaction rate constant} \\ C_0 &= \textit{initial concentration} \\ C &= \textit{concentration at time, } t. \end{aligned}$$
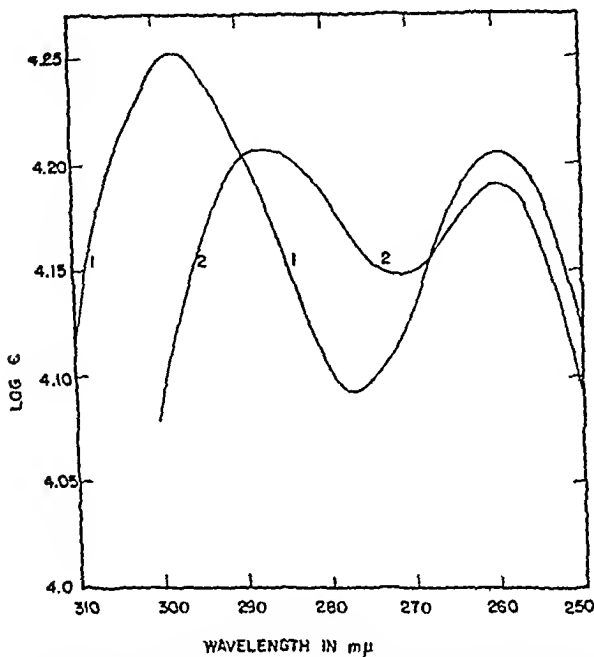
FIG. 3. ULTRAVIOLET ABSORPTION CURVES OF 2-SULFANILAMIDO-4-METHYL-5-CARBOXYTHIAZOLE AND 2-SULFANILAMIDO-4-METHYLTHIAZOLE

3.74×10^{-4} M solutions in 0.05 M phosphate buffer at pH $= 7.0$. Curve 1, 2-sulfanilamido-4-methyl-5-carboxythiazole before autoclaving; Curve 2, solution of Curve 1 after autoclaving and 72-hour incubation at 37°C. This curve is identical with that obtained for 2-sulfanilamido-4-methylthiazole before and after autoclaving.

The rate constants, with t in hours, and corresponding half-lives were as follows: sulfacarboxythiazole, $k = 6.35 \times 10^{-4}$ hr.$^{-1}$, $t_{1/2} = 1090$ hrs.; 2-sulfanilamido-4-methyl-5-carboxythiazole, $k = 6.65 \times 10^{-3}$ hr.$^{-1}$, $t_{1/2} = 104$ hrs.

In the light of these decomposition rates and the results in table 1, it is at once obvious that it is not necessary to assume any activity due to the carboxy forms of sulfacarboxythiazole and the sulfamethylcarboxythiazole. Also, the presence of sulfathiazole as an impurity to the extent of only 0.4% would account for the observed activity of 2-sulfanilamido-4-carboxythiazole.

On the basis of acid strengths of the amide hydrogen (second acid constant)

the carboxy forms of these compounds should be very active (13). pK_a's obtained by alkali titration and not corrected for dilution were: sulfacarboxythiazole, $pK_a(1) = 3.4$, $pK_a(2) = 7.0$; 2-sulfanilamido-4-methyl-5-carboxythiazole, $pK_a(1) = 3.4$, $pK_a(2) = 7.7$; and 2-sulfanilamido-4-carboxythiazole, $pK_a(1) = 3.3$, $pK_a(2) = 6.9$. However, at the pH of the bacteriostatic tests (7.2), the carboxyls of all of these compounds are better than 99 per cent ionic. If one assumes that such ionic forms do not penetrate bacteria (14, 15) then it is not surprising that the carboxy compounds, as such, were found to be relatively inactive.

Blood concentrations in mice[1]: Comparative blood concentration-time values following a single oral dose of 0.5 grams per kgm. in mice are given in table 2. Sulfacarboxythiazole blood levels were very low as was the case with sulfathalidine. Sulfathiadiazole blood levels were somewhat higher than those

TABLE 2

Drug concentrations in blood of mice after a single oral dose of 0 5 gm./kgm in 10% acacia solution

COMPOUND	DRUG CONCENTRATION IN BLOOD AT HOUR				
	1	2	4	8	24
	mgm %	*mgm %*	*mgm %*	*mgm. %*	*mgm. %*
Sulfathiazole	12 0	7 4	4.6	4.6	0.4
Sulfadiazine	18 9	20.0	18.9	10 3	0.4
Sulfathiadiazole	6.1	2 8	2.0	1.2	0.7
Sulfaguanidine	2 6	1 7	0 5	0	0
Carboxythiazole	1.0	0.5	0 3	0.3	0
Sulfathalidine	0.7	0.6	0 6	0.3	0.3

Each determination (Bratton and Marshall method) was made as free drug on pooled tail blood samples from ten mice Mice were without food for 18 hours prior to administration of drug.

for sulfaguanidine, but appreciably lower than those obtained with sulfathiazole and sulfadiazine. The fact that sulfathiadiazole gave blood levels higher than those of sulfaguanidine was not considered to be necessarily disadvantageous with respect to usefulness in treating bacillary dysentery, as will be discussed later in this paper.

Blood levels which were more or less constantly maintained in mice as a result of continual feeding on drug-diets for seven days are given in table 4. Under these conditions, the sulfacarboxythiazole and sulfathalidine levels were again found to be very low, while those of sulfathiadiazole and sulfaguanidine were higher, but not as high as the sulfathiazole and sulfadiazine values.

Bacteriostatic activity: The dysentery strains used in this study were obtained

[1] We wish to thank Miss Dorothea Babbitt for assistance in determinations on blood concentration values.

through the courtesy of Dr. A. J. Weil of the Lederle Laboratories. The test procedure has previously been described in detail (16). The activity of each drug was titrated by making serial two-fold dilutions in 5 ml. volumes of Tryptiease-Soy Phosphate Broth (Baltimore Biological Laboratory) buffered at pH 7.2. After autoclaving, each tube in each drug series and control tubes without drug were inoculated with 0.2 ml. of a 10^{-6} broth dilution of a 22-hour broth test culture. The relative values in table 3 were confirmed in repeated comparisons.

On the basis of bacteriostatic endpoints for all four drugs, the dysentery strains may be divided into a resistant group and a relatively susceptible group, with six strains in each group. In view of a clinical study (17) which indicated that Flexner varieties of *Shigella paradysenteriae* were more sensitive than Sonne

TABLE 3

Comparative activity of sulfonamides against dysentery strains in 0.4% peptone broth

DYSENTERY STRAINS		MINIMAL CONCENTRATION OF DRUG IN MGM % REQUIRED TO PREVENT VISIBLE GROWTH			
		Sulfathiazole	Sulfadiazine	Sulfaguanidine	Sulfathiadiazole
Sonne	B-151	256	>256	>256	256
Flexner.	5733	128	256	>256	256
Shiga	70-151	128	256	128	512
Sonne	B-152	64	256	>256	256
Schmitz	B-161	64	256	>256	256
Shiga	B-111	16	>256	>256	64
Flexner	63-143-Z	4	8	128	16
Flexner	63-143-V	1/4	1/2	8	1
Schmitz	B-162	1/8	1	16	2
Flexner	63-143-X	1/8	1	16	2
Flexner	63-143-Y	1/8	1/2	8	1
Flexner	63-143-W	1/64	1/64	2	1/2

Test medium. 0.4% Trypticase-Soy-Phosphate Broth buffered at pH 7.2.
Inoculum. 50 ± 20 bacteria per ml.
Incubation 48 hours at 37°C.

varieties to sulfonamide treatment, it is of interest that, in our tests five of the six Flexner strains were relatively susceptible to one or more drugs, whereas each of the two Sonne strains were relatively resistant to all of the drugs.

Under our test conditions, five strains were resistant to sulfaguanidine concentrations approximating its highest solubility. Two of these strains were also resistant to the highest concentration of sulfadiazine which could be obtained at pH 7.2. Inhibition of the sulfaguanidine-resistant strains with high concentrations of the other drugs was readily accomplished by dissolving their sodium salts in the test medium and adjusting the pH to 7.2.

Against the sulfonamide-susceptible strains, sulfathiadiazole was from four to eight times as active as sulfaguanidine.

Anticoliform activity in mice: Full details of the method used for this compari-

son have been given in a previous paper (18). In brief, mice (Carworth CFCW strain) were arranged in groups which were equivalent with respect to fecal coliform counts on the basis of preliminary determinations. Each group was then treated with a different drug-diet. Each mouse in each group was kept in an individual cage and allowed to feed on drug-diet for seven days at which time final coliform counts were made. Coliform counts per unit volume of a standard stool suspension were determined by serial ten-fold dilutions in lactose broth incubated at 44°C. Drug activity was expressed as the difference between coliform counts at the beginning and at the end of drug-diet treatment.

In table 4 the results for six drugs, together with the data on control animals, are summarized and listed in order of decreasing anticoliform activity. With each drug, it is evident that, on the average, the pre- and post-treatment coliform

TABLE 4

Sulfonamide activity against coliform bacteria in mice

DRUG	PER CENT IN DIET	NUM- BER OF MICE	DAILY INTAKE		DRUG CONCEN- TRATION		LOGARITHMIC COLIFORM COUNT PER 9 CC. OF STOOL SUSPENSION		
			Food	Drug	Blood	Stool	Be- fore treat- ment	After treat- ment	Reduction
			gm	gm / kgm	$mgm / 100\ cc$	$mgm / 100\ gm$			
Sulfathiazole	1.0	63	4.4	2 2	9.2	140	6.5	1.5	5.1 ± 0.3
Sulfadiazine	0.5	17	4.4	1 1	26.6	910	6.3	1.8	4.5 ± 0.5
Sulfathiadiazole .	1.0	66	4.7	2.4	4.1	580	5.8	1.5	4.3 ± 0.3
Sulfaguanidine	1.0	63	4.7	2.4	2.6	1100	5.7	2.6	3.1 ± 0.4
Sulfacarboxythiazole	1 0	43	4 6	2.3	1.1	2300	5.7	2.7	3.0 ± 0.4
Sulfathalidine	1 0	25	5 0	2.5	0.5	1250	5.4	3.4	2.0 ± 0.4
Control Diet		117	4 6				5.8	5.7	0.1 ± 0.2

Figures above are mean values. Reductions in logarithmic counts are given with their standard errors. In each case, the mice were fed on drug-diet for seven days. Blood concentrations were determined as free drug on the third day; stool concentrations of each drug were calculated on the basis of an average value of 0.275 grams of wet stool per 100 cc. of standard stool suspension.

counts differed significantly. It is also evident that each of the drugs produced, on the average, a reduction in coliform count which was significantly greater than the reduction which occurred in untreated mice. Thus, the anticoliform effect ranged from about a hundred-fold reduction with sulfathalidine to about a hundred thousand-fold reduction with sulfathiazole. On the basis of average reduction in count, sulfathiazole, sulfadiazine and sulfathiadiazole were all more active than sulfaguanidine, sulfacarboxythiazole and sulfathalidine.

DISCUSSION. One aspect of the bacillary dysentery problem is concerned with the treatment of mild cases and the prevention of epidemic spread by eliminating pathogenic organisms from convalescents and carriers. In these individuals, the bacteria presumably reside chiefly in the lumen of the intestinal tract and the situation calls for an active drug which is highly soluble, but slowly absorbed

and rapidly excreted in the urine, in order to minimize toxic effects which might occur in the absence of strict medical supervision.

Another part of the dysentery problem consists of treating cases, of varying severity, in which the pathogenic bacteria are probably not only in the lumen of the intestinal tract, but also in the lining tissue, often covered by a muco-purulent exudate. Under these conditions, optimal results should be achieved by treatment with a drug which is maintained in an effective concentration in the blood as well as in the intestinal contents. Thus, although a low concentration of drug in the blood has been emphasized as a required characteristic, it is quite possible that this has been over-emphasized and that, in many cases, better results would be obtained with a drug which is absorbed to a moderate extent (17).

Sulfadiazine and sulfathiazole are examples of well absorbed drugs which have been used with a certain amount of success for the treatment of bacillary dysentery (17, 19, 20, 21, 22). These drugs were highly active under our test conditions (tables 3 and 4). But they are absorbed and excreted in a manner which results in rather high concentrations in the blood (tables 2 and 4) and, furthermore, they are soluble to a relatively low degree at intestinal and urinary pH's (fig. 1).

N⁴-Succinylsulfathiazole and N⁴-phthalylsulfathiazole (sulfathalidine) are examples of very poorly absorbed drugs which have been used for treating enteric infections (17, 22, 23, 24, 25). Apparently one of the drawbacks encountered with the succinyl compound is that it tends to be ineffective in the presence of a watery diarrhea (25). Sulfathalidine, as the sodium salt, is highly soluble at a pH of 7.6 (26). However, according to Poth (25), there is no tangible evidence to show that either succinyl- or phthalylsulfathiazole possesses antibacterial properties which are not entirely dependent upon the presence of sulfathiazole itself, presumably resulting from hydrolysis. This is in agreement with our results (18). Thus, in their inactive form these compounds are highly soluble and very poorly absorbed; and they appear to be effective by means of a slow breakdown to sulfathiazole.

Sulfacarboxythiazole appears to be another type of compound which owes its activity to the release of an active component. It is highly soluble at pH's which may be expected in the intestinal contents (fig. 1); it is poorly absorbed (9); and it presumably releases sulfathiazole at a rate and to an extent sufficient to produce an antibacterial effect about equal to that of sulfaguanidine (table 4).

Sulfaguanidine has been used with considerable success both therapeutically and prophylactically (17, 19, 23, 27, 28, 29, 30). It appears to have been a relatively safe drug for prophylactic use, due to the fairly low blood levels which result from large doses. But it is soluble to only a moderate degree (fig. 1) and it is not as active as sulfathiazole or sulfadiazine (tables 3 and 4).

Sulfathiadiazole, on the basis of the comparative data obtained in the present investigation, appears to be a drug with interesting possibilities for use in bacillary dysentery. It is more active than sulfaguanidine both *in vitro* and *in vivo* (tables 3 and 4); it does not appear to owe its activity to a decomposition product, as do sulfathalidine and sulfacarboxythiazole; and, finally, it is highly soluble at

intestinal and urinary pH's (fig. 1). Preliminary pharmacological studies (31) indicate that, following oral dosage, the absorption and excretion of the compound occurs in such a manner as to result in high concentrations in the intestinal contents, moderately low concentrations in the blood and relatively high but, due to its solubility, probably safe concentrations in the urine.

The final answer to the question as to which sulfanilamide derivative may be most useful in the treatment of bacillary dysentery can only be determined on the basis of carefully controlled clinical and field trials. In addition to this question, there remains the important problem of finding new drugs which will be effective against sulfonamide-resistant strains of Shigella.

CONCLUSIONS

1. Sulfacarboxythiazole (2-sulfanilamido-5-carboxythiazole) was about as active as sulfaguanidine against twelve dysentery strains *in vitro* and against coliform bacteria in mice. With respect to solubility, absorption and mode of action, sulfacarboxythiazole appeared to be similar to sulfathalidine (N^4-phthalyl-sulfathiazole).

2. Sulfamethylcarboxythiazole (2-sulfanilamido-4-methyl-5-carboxythiazole) decarboxylates quite readily to sulfamethylthiazole. Consequently, further investigation of this compound, as a possible chemotherapeutic agent, does not appear to be warranted.

3. 2-Sulfanilamido-4-carboxythiazole does not decarboxylate readily but is relatively inactive.

4. Sulfathiadiazole (2-sulfanilamido-1,3,4-thiadiazole), when compared with sulfaguanidine, was found to be about ten times as soluble at pH 6.5; from four to eight times as active against dysentery strains *in vitro;* and more active against coliform bacteria in mice. These properties, together with preliminary data on its absorption in mice, indicate that sulfathiadiazole is, at present, one of the most promising compounds available for trial in the treatment and prophylaxis of bacillary dysentery.

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THE INHIBITORY EFFECT OF ATABRINE AND SOME ACRIDINE DERIVATIVES UPON ACID-FAST BACILLI IN VITRO*

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In 1917 Lewis (1) studied the effect of a large number of dyes upon the tubercle bacillus and found acridine orange exhibited a high degree of inhibitory action. Smith (2) corroborated Lewis' findings on acridine orange and showed that acriflavine, proflavine and acridinium yellow possessed a high degree of inhibition. A preliminary report (3) by us has shown that atabrine and two acridine derivatives had a marked inhibitory effect upon the growth of seven species of acid-fast bacilli. Lawrence (4) investigated the effect of quinine, atabrine and some substituted acridine compounds on the gram negative bacilli *in vitro* and Campbell and Gilchrist (5) compared the anti-bacterial efficiency of atabrine, sulfapyridine and sulfanilamide against various pathogenic organisms, not including the mycobacteria. The present paper presents the results of further experiments using atabrine and fourteen acridine derivatives.[1]

The strains used were obtained from the American Type Culture Collection with the exception of one strain of *H 37* from Trudeau Sanatorium and another isolated from a case of pulmonary tuberculosis in this hospital which, after frequent subculture, grew rapidly. Aqueous stock solutions of the various compounds were made by dissolving accurately weighed samples in distilled water and storing in Corning "low actinic", glass stoppered bottles in the ice box. Sterile filtrates were obtained by using Corning UF sintered glass filters. It was noticed that a slight amount of color was retained by this filter after washing. The Boerner metal filter was unsatisfactory for this purpose as the filter discs removed both the color and activity from atabrine. A 1:10000 dilution of atabrine showed no color after filtration and upon plate cup assay exhibited no activity, whereas the unfiltered solution had a very definite greenish color and upon assaying produced a zone of inhibition of greater than 30 mm. Two series of sterile plates were prepared using 20 cc. of Difco nutrient agar in one and the same amount of 2% glycerine agar in the other. Care was taken to pour the plates on a level surface so that the agar was the same depth throughout. These plates were inoculated with a 2 mm loopful from young cultures of the various strains. By means of a tantalum spatula the inoculum was spread as evenly as possible over the surface of the agar. The cup assay method (6, 7) was used for this study. Four sterile penicylinders were placed on each plate and duplicate assays were made. In many instances the tests were rechecked. Daily measurements of the zones of inhibition were recorded. The zone diameters of the fast growing strains reached a maximum usually at the end of 72 hours incubation at 35°C. Table 1 shows the inhibitory effect of atabrine and two acridine derivatives upon seven acid-fast species.

It may be seen from table 1 that the glycerine agar plates have smaller zones of inhibition than the nutrient agar plates. Of the three acridine derivatives

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atabrine appears to be the least active. Derivative No. 3 shows slightly greater inhibitory activity for *M. phlei*, *M. berolinensis*, and *M. leprae*. All three derivatives show approximately equal activity against *M. tuberculosis*, *M. avium*, and *M. chelonei* on nutrient agar but on the glycerine agar plates of

TABLE 1

Inhibitory effect of three acridine derivatives upon seven acid fast species

Plate cup assay method

ORGANISM		DILUTIONS											
		1:10000			1:20000			1:40000			1:80000		
		Drug no.*			Drug no.*			Drug no.*			Drug no.*		
		6	1	2	6	1	2	6	1	2	6	1	2
		Zone diameter in mm. at 72 hrs.											
Mycobacterium avium 7992 A.T.C.C.	N	>30	30	>30	30	28	30	24p	21	26	19p	16p	18p
	G	15	24	20	?	20	20	0	12	18		0	12
Mycobacterium phlei 355 A.T.C.C.	N	25	30	30	23	27	30	15	23	27	0	15	25
	G	30p	30	30	25p	27	25	?	19	24	0	14	18
Mycobacterium berolinensis 20 A.T.C.C.	N	>30	30	>30	>30	29	>30	24p	28	>30	15p	24p	27
	G	23	24	26	20	21	24	14p	15p	22	0	?	12p
Mycobacterium chelonei 114 A.T.C.C.	N	30	30	27	27	27	25	25	24	24	18	17	18
	G	23	26	26	17	24	23	0	18	21		13	16
Mycobacterium smegmatis 101 A.T.C.C.	N	30	25	30	26	23	24	24	22	22	14p	18	18
	G	20p	25	30	?	24	26	0	19	23	0	15	15
Mycobacterium leprae 4243 A.T.C.C.	N	>30	27	>30	23	25	30	16p	20	27	0	16	20
	G	27	27	22	23	24	18	16	16	16	0	0	0
Mycobacterium tuberculosis Human†	N	>30	27	30	30	25	27	25	24	26	16	21	21
	G	25	25	25	21	22	24	16	18	21	12p	12	14

* Drug number

    6 = 2-Methoxy-6-chloro-9-(1 Methyl-4-diethyl amino butyl) amino acridine hydro-chloride

    1 = 2-Methoxy-6-chloro-9-(γ-N-piperidylpropyl amino) acridine dihydrochloride

    2 = 2-Methoxy-6-chloro-9-(δ-diethylaminobutyl) amino) acridine dihydrochloride

† Isolated here from a case of pulmonary tuberculosis.

N = Nutrient agar

G = 2% Glycerine nutrient agar

p = Partial inhibition refers to zones which are definite but not completely clear

atabrine at 1:40000 dilution *M. avium*, *M. chelonei*, and *M. smegmatis* showed no inhibition, while the plates of *M. tuberculosis* exhibited zones of 16 mm.

After 18 hours incubation of the nutrient agar plates inoculated with these various strains, smears were made and glycerine agar slants streaked from the cleared zones around the cylinders of the 1:5000 dilution of atabrine. In the same manner controls were made from the areas unexposed to the drug. At the

end of five days all of the subcultures were negative except for *M. leprae* which showed a few scattered colonies. All of the controls grew normally, the slants being almost completely covered. The stained smears made from the cleared areas showed a partial loss of acid fastness, fewer granules and the cells were two to eight times as long and slightly wider than those unexposed to the drug. The avian strain showed long branching filaments and *M. berolinensis* was the only preparation to show increased acid-fastness after exposure to atabrine.

The sensitivity to atabrine of two slow growing strains, *H 37* and *M. bovis*, was estimated by inoculating 2% glycerine agar slants which contained graded dilutions. This method was employed as the agar plate cup assay technique proved unsatisfactory for slow growing strains. After inoculation the tops of the cotton plugs were flamed and pushed into the neck of the tube. The tube was then stoppered with a sterile one-holed rubber stopper. This prevents rapid drying and allows for gaseous exchange. Duplicate slants of the dilutions 1:10, 1:20, 1:40 and 1:50 thousand were inoculated heavily with a 2 mm. loopful of a three weeks old luxuriantly growing culture. This was spread as evenly as possible over the surface. The inoculum was easily visible. Controls were also inoculated in the same manner.

At the end of two and one-half months the 1:10000 tubes showed no growth for either the bovine or human strains. At dilution 1:20000 three of four tubes of the human strain were negative; the fourth tube had only one small colony. All four tubes of the bovine strain showed scattered, poor growth at the end of a month. A transfer was made from one of these tubes and good growth occurred by the end of two and one-half months. Dilutions 1:40000 and 1:50000 showed good growth noticeably stained by the atabrine. The controls showed normal growth at the end of four weeks. The *H 37* strain at dilution 1:40000 showed only two small colonies in each tube at the end of three and one-half months. The 1:50000 dilution showed scattered growth with small stained colonies at the end of this period while the controls at the end of four weeks showed the usual heavy growth.

A freshly isolated strain from the spinal fluid of a patient was also tested. At the end of three weeks the controls had grown luxuriously while the 1:20000 dilution showed only a few minute scattered colonies.

The comparative activity of fifteen acridine derivatives upon the rapidly growing strain of *M. tuberculosis* is summarized in table 2. The cup assay method was used. From table 2 it can be seen that drugs No. 1 and 2 exhibit approximately equal activity on both the nutrient and 2% glycerine agar plates; numbers 3, 4, 5, and 6, in the order of their decreasing activity, show larger zones of inhibition on nutrient agar than on glycerine. Drugs No. 7, 11, 12, 13 and 14 are relatively inactive on glycerine, with slight or no inhibition at 1:20000 dilution, while No. 8, 9, and 10 are relatively poor on both media, showing only partial inhibition on nutrient agar and none on glycerine agar at 1:20000 dilution. Of all of these compounds, No. 15 appears to be the most active on both media with No. 3 next in order.

Subcultures were made from some of these plates by inoculating scrapings

TABLE 2

Inhibitory effect of atabrine hydrochloride and fourteen acridine derivatives upon Mycobacterium tuberculosis

Plate cup assay method

NO.	DRUG		DILUTIONS			
			1:10000	1:20000	1:40000	1:80000
			Zone diameter in mm. at 72 hrs.			
1	2-Methoxy-6-chloro-9-(γ-N-piperidylpropyl amino) acridine dihydrochloride	N / G	27 / 25	25 / 22	23 / 18	17 / 12
2	2-Methoxy-6-chloro-9-(δ-diethylaminobutyl amino) acridine dihydrochloride	N / G	30 / 25	27 / 24	22 / 21	18 / 14
3	2-Methoxy-6-chloro-9-(γ-ethylisopropylamino-propylamino) acridine dihydrochloride	N / G	30 / 25	30 / 20	27 / 16	24 / 0
4	2-Methoxy-6-chloro-9-(1'-diethylamino-5'-hexyl-amino) acridine dihydrochloride	N / G	30 / 19	27 / ?	24 / 0	21 / 0
5	2-Methoxy-6-chloro-9-(γ-N-(α'-pipecolino)-propyl-amino) acridine dihydrochloride	N / G	30 / 22	28 / 20	24 / 15	20 / ?
6	2-Methoxy-6-chloro-9-(1 methyl-4-diethylamino-butyl) amino acridine hydrochloride	N / G	32 / 25	30 / 20	25 / 16	15 / 12p
7	2-Methoxy-6-chloro-9-(1'-n-butylethylamino 4'-pentylamino) acridine dihydrochloride	N / G	30 / 15	26 / 0	19 / 0	12 / 0
8	2-Methoxy-6-chloro-9-(γ-di-n-butylaminopropyl-amino) acridine dihydrochloride	N / G	25 / 15	22p / 14	19p / 0	0
9	2-Methoxy-6-chloro-9-(β-(β'-methoxy)ethyliso-propylaminoethylamino) acridine dihydrochloride	N / G	23 / 14p	15p / 0	0	0
10	2-Methoxy-6-chloro-9-(β-ethyl-2'-pentylamino-ethylamino) acridine dihydrochloride	N / G	19p / 11	15p / 0	?	0
11	9-β-Hydroxyethylaminoacridine hydrochloride	N / G	30 / 20	30 / ?	30	15
12	9 Benzylaminoacridine hydrochloride	N / G	30 / 23	30 / 15	30 / 0	15
13	9-Ethylaminoacridine hydrochloride	N / G	30 / 18	30 / 13	30 / 0	24 / 0
14	9 (4'-Diethylamino-1'-methyl) butylaminoacridine dihydrochloride	N / G	30 / 16	30 / ?	28	22
15	3-Chloro 9-(4' Diethylamino-1'-methyl) butyl-aminoacridine dihydrochloride	N / G	30 / 27	30 / 24	35 / 21	28 / 15

N = Nutrient agar
G = Glycerine agar
p = Partial inhibition

from the zones of inhibition onto pairs of glycerine agar slants. Controls were made in a similar manner from the areas unexposed to the drug. The subcultures of the 1:40000 dilution area of No. 11 taken at the end of sixty-six hours showed no growth after five weeks of incubation. The 1:80000 area showed one colony. No. 5, tested in exactly the same manner, gave negative results on all slants except in the case of one slant from the 1:40000 dilution which developed a single colony. One of the glycerine agar slants from the 1:40000 dilution of No. 14 incubated for two weeks was negative, the other showed one colony. The subcultures from 1:80000 dilution showed heavy growth. After 120 hours of incubation this plate gave sterile subcultures from the 1:80000 dilution. Subcultures made after 48 hours' incubation of the plate with No. 15 drug showed heavy growth from the area of 1:80000 dilution while those from the 1:40000 area showed no growth after five weeks. The subcultures after five days incubation from another plate of 1:80000 and 1:100,000 dilutions of this drug were sterile at the end of five weeks. The controls grew normally. Thus, it may be seen that the subcultures at 1:40000 taken after 66 hours' incubation showed no growth at the end of five weeks. In dilutions as high as 1:80000, if the subcultures were taken at the end of 66 hours, growth occurred. However, if the plates were incubated 120 hours sterile subcultures were obtained.

MacLeod's and Mirick's bacteriostatic studies on pneumococci (8) have described a technique whereby most of the sulfonamide inhibitor contained in plain peptone broth may be removed by boiling the medium with charcoal at an acid reaction, and have also stated that the growth promoting properties of the broth are greatly improved by this process due to the simultaneous removal of substances which inhibit growth. It seemed of interest then to find out if the growth of acid fast organisms on nutrient broth or agar would be affected when the medium was treated in this manner and if the inhibitory effect of atabrine would be altered.

Nutrient broth and broth adsorbed by the above method were tubed and sterilized in 5 cc. amounts. Atabrine was added to make a dilution of 1:25000. The tubes were inoculated with a 2 mm. loopful of growth from young agar slant cultures of the following rapidly growing strains: *M. tuberculosis*, *M. avium*, *M. chelonei*, *M. phlei*, *M. berolinensis* and *M. leprae*.

At the end of 48 hours the controls grew equally well in both the adsorbed and unadsorbed broths. The atabrine tubes of adsorbed and unadsorbed broth inoculated with the human, avian, and phlei cultures showed no growth at the end of 25 days. The tubes inoculated with *M. chelonei* and *M. berolinensis* showed complete inhibition in the adsorbed broth whereas there was growth in the bottom of the tubes of nutrient broth, with *M. berolinensis* also forming a delicate pellicle. *M. leprae* grew equally well in all the tubes.

The effect of the adsorption of nutrient agar with and without the addition of 2% glycerine was next studied. *M. avium*, *M. phlei*, *M. berolinensis*, *M. chelonei*, *M. leprae*, *H 37*, *M. bovis*, and *M. tuberculosis*, the quickly growing strain, were inoculated as previously described onto slants of adsorbed and nonadsorbed nutrient and glycerine agar containing 1:10000, 1:20000, 1:40000 dilutions of atabrine. At the end of three weeks *M. avium* had grown only on the nonadsorbed glycerine agar at 1:20000. At 1:40000 dilution this strain showed no growth on the adsorbed glycerine agar slants but grew well on the others. *M.*

leprae grew at 1:10000 on the nonadsorbed glycerine agar and at dilution 1:20000 grew on both the adsorbed and nonadsorbed glycerine agar. *M. tuberculosis*, the quickly growing strain, did not grow on the adsorbed media but showed growth at all dilutions on nonadsorbed glycerine agar and at 1:40000 on nonadsorbed nutrient agar. The *H 37* strain did not grow on any of the slants at dilutions 1:10000 and 1:20000. At the higher dilution it grows on all the media. However, the growth on the adsorbed and nonadsorbed agar is better with glycerine than without it. *M. bovis* grew on both kinds of glycerine agar at 1:20000 and all four types of media at 1:40000. The controls of the six quickly growing strains showed the usual-species growth on all of the slants, with possible slight differences in extent of growth. In the case of *H 37* the glycerine agar slants, adsorbed and nonadsorbed (six tubes each) showed normal growth on all of the nonadsorbed slants at the end of a month, while three of the adsorbed slants were negative and the other three showed thin spreading growth. The slants without glycerine showed the same effect but to a lesser extent. *M. bovis* likewise showed good growth on the nonadsorbed but a thin veil-like dull type developed on the adsorbed media. Furthermore, it was found that the addition of sterile 20% human plasma to the agar plates did not alter the atabrine titer toward *M. tuberculosis*.

The sensitivity to atabrine of these eight strains with the exception of *M. chelonei* and *M. bovis* is less on nonadsorbed glycerine agar than on any of the other media. Without atabrine, *H 37* and *M. bovis* exhibited a glycerinophilic character, growing better on both the adsorbed and nonadsorbed glycerine agar slants. Adsorption of the media for all of these acid-fast organisms does not improve its growth promoting properties.

An interesting phenomenon observed in these plate assays was the delayed appearance of the zones of inhibition with some derivatives. For example, the atabrine plates and those of 2-methoxy-6-chloro-9-(γ-di-*n*-butyl-amino propylamino) acridine dihydrochloride show this phenomenon most strikingly. At the end of 24 hours the growth of *M. tuberculosis* covers the surface of the agar plate. After 48 hours the areas of growth are clearing around the penicylinders. In the next 24 hours the zones become clear and may increase in size, while the growth on the rest of the plate increases. In contrast, other derivatives, such as, 2-methoxy-6-chloro-9-(γ-N-piperidylpropylamino) acridine dihydrochloride, 2-methoxy-6-chloro-9-(δ-diethylaminobutylamino) acridine dihydrochloride and 3-chloro-9-(4'-diethylamino-1'-methyl) butylaminoacridine dihydrochloride, show very clear zones at the end of 24 hours, increasing to a maximum at 48 to 72 hours. In both these instances the zones remain clear indefinitely. This suggests that some of the derivatives have a more immediate effect than others upon *M. tuberculosis*.

CONCLUSIONS

These *in vitro* studies show that at least six of the acridine derivatives tested exhibit marked inhibition of *M. tuberculosis* on both glycerine and nutrient agar. Of the many compounds showing antibacterial activity against *M. tuberculosis*, atabrine perhaps has been more extensively investigated pharmacologically than any other. Its non-toxicity for humans is well established. As some of these other derivatives, notably 3-chloro-9-(4'-diethyl amino-1'-methyl) butyl-aminoacridine dihydrochloride, show a greater inhibitory effect than atabrine

towards *M. tuberculosis in vitro* it would seem of interest to investigate these particular compounds further.

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THE SPIROCHETICIDAL AND TRYPANOCIDAL ACTION OF ACID-SUBSTITUTED PHENYL ARSENOXIDES AS A FUNCTION OF pH AND DISSOCIATION CONSTANTS

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A series of acid-substituted phenyl arsenoxides has now been studied with respect to spirocheticidal (1) and trypanocidal (2) activity. With only a few exceptions, the acidic groups caused a marked decrease in the activity of the parent phenyl arsenoxide. This inhibition was apparently not referable to an effect on the dissociation of the —AsO or —As(OH)$_2$ group, since the unsubstituted phenyl arsenoxide gave the same titration curve in the appropriate pH range (pH 7–12) as its acid-substituted derivatives (1). Alternatively, the inhibitory effect of acidic substituents might be related to their own ionization. It follows from the dissociation constants of those substituents (cf. table 1) that almost all would be more than 99 per cent ionized at pH 7.4; and one could explain their observed inhibitory effect if the ionized salts of these compounds were relatively inactive as compared with the undissociated free acids. In that case, one would expect the activity at any pH to be a function of the pK of the compound, those with a lower dissociation constant (higher pK) having more of the non-ionized form, and thus, a higher activity. In a small series of acid-substituted compounds, previously reported data (1) did suggest such a correlation between the pK of the substituent group and the treponemicidal activity. However, the correlation was partial only, there were several exceptions, and other factors were clearly complicating the picture.

It will be shown in the present paper that this concept is nevertheless fundamentally correct. The direct parasiticidal activity of acid-substituted phenyl arsenoxides is apparently determined by at least four factors: the generally slight, but variable, activity of the ionized salt; the regularly high activity of the undissociated free acid; and the proportion of the dissociated (relatively inactive) and undissociated (highly active) forms, determined by the pK of the acidic substituent and the pH at which the drug was tested. The effect of the organisms themselves in promoting activity by binding the non-ionized, active form of the compound is discussed in the text.

METHODS AND MATERIALS. The preparation of the compounds discussed in the present paper has been described elsewhere (3) (4), as has the method used for the determination of their dissociation constants (1).

The method used for the assay of spirocheticidal (5) and trypanocidal activity (2) in vitro has also been previously described. The method involved the determination of the concentration of compound necessary to immobilize 50 to 60 per cent of the organisms, compared to a reference compound (phenyl arsenoxide) simultaneously tested, with the same suspension and under the same conditions. The suspension of S. pallida was obtained by

TABLE 1

The inhibitory effect of acidic substituents on the spirocheticidal and trypanocidal activity of phenyl arsenoxides

SUBSTITUENT GROUPS (RC₆H₄AsO OR R₁R₂ (C₆H₄AsO))	SPIROCHETICIDAL ACTIVITY PER MOLE[a]	TRYPANOCIDAL ACTIVITY PER MOLE[a]	pK (1)
Unsubstituted phenyl arsenoxide........	100	100	
o-COOH—	28	3.2	5.55
m-COOH—..	13		4.25
p-COOH—........	6.7	0.5	4.0
o-SO₃H—.........	1.5		
p-SO₃H—..	3.3	0.06	2.0±
p-CH₂COOH—	4.2	4.7	4.35
p-(CH₂)₂COOH—	4.1	2.8	4.7
p-(CH₂)₃COOH—		54	4.9
p-(CH₂)₄COOH—		27	5.2[b]
p-(CH₂)₅COOH—	22	9.0	5.35[b]
m-CH=CHCOOH— .. .	3.9		
p-CH=CHCOOH—.....	17	2.0	
p-OCH₂COOH—......	5.2	4.5	3.3
p-CHOHCOOH—	2.8	0.5	
p-(C₂H₅)CHCOOH— ..		10	4.8
p-NHCO(CH₂)₂COOH— ..	6.4		4.75
p-CONHCH₂COOH—. .	0.7	0.2	
3-NO₂-4-COOH—	18		2.6
3-NO₂-4-SO₃H— .	3.5		
2-COOH-4-CONH₂—	2.6		
3-NH₂-4-(CH₂)₂COOH—		10.25	5.35
2-COOH-3-NH₂— .		23	
3-NH₂-4-COOH—	20	4.0	4.7
3-NH₂-5-COOH—		5.0	
2-NH₂-6-COOH—		2.4	
2-COOH-5-NH₂—		1.8	6.45
2-COOH-4-NH₂—		0.7	
2-NH₂-3-COOH—		0.6	
2-NH₂-4-COOH—		0 6±	
2-NH₂-5-COOH—		0.6	

[a] Referred to that of unsubstituted phenyl arsenoxide as 100. In these early experiments, the pH was unfortunately not rigorously controlled, but varied in the range 6 8 to 7.4. To that extent the activities listed in the table represent approximations only (cf. fig 5). The further inaccuracy introduced by the varying organisms used in the assay is discussed in the text.

[b] Titration inaccurate because of the precipitation of free acid in acidic range.

[c] Titration was prevented by precipitation of free acid on addition of small amounts of dilute HCl to the sodium salt. The fact that precipitation began at pH 6 suggests a fairly high pK.

TABLE 2

Illustrating the method used in determining the effect of pH on the trypanocidal activity of phenyl arsenoxides

(Data of this table appear as filled circles in fig. 1)

Eleven duplicate rows of tubes were prepared, each containing a) varying amounts of a varying dilution of the p-$(CH_2)_2COOH$ phenyl arsenoxide, brought to a total volume of 0.4 cc. with 0.85 per cent NaCl; b) 0.4 cc. of the various buffer mixtures indicated below, and c) 0.4 cc. of a suspension of trypanosomes (T. equiperdum) in 40 per cent serum (i.e., serum + 1½ volumes of 0.85 per cent NaCl). The method of preparing the trypanosome suspension, free of red blood cells, has been described elsewhere (2). The final trypanosome count in the drug-buffer-organism suspension was approximately 10 x 10⁶ per cc.

After 2 hours at room temperature, the proportion of motile organisms was determined, as indicated below. The amount of drug necessary to immobilize 55% of the organisms was obtained by interpolation. That amount, compared to the amount of a 1:2,000,000 dilution of the reference compound (unsubstituted phenyl arsenoxide) simultaneously tested, gave a measure of the gravimetric activity of the compound. In setting up the experiment, care was taken to allow a measured interval of 5 to 10 minutes between the addition of organisms to successive rows of the assay. The same interval was allowed in determining the proportion of survivors, so that the trypanosomes were left in contact with the drug for the same interval of time throughout.

BUFFER MIXTURE (TOTAL VOLUME 0.4 cc.)				pH OF FINAL DRUG-BUFFER-SERUM TRYPANOSOME MIXTURE (1.2 cc.)	CONCN. OF ARSENICAL USED (p-(CH₃)₂COOH PHENYL ARSENOXIDE)	CC. OF ARSENICAL SOLUTION IN TOTAL OF 1.2 CC.							CC. OF ARSENICAL NECESSARY TO IMMOBILIZE 55% OF ORGANISMS (45% STILL MOTILE)	TRYPANOCIDAL ACTIVITY REFERRED TO PHENYL ARSENOXIDE AS 100	
NaOH M/10	NaHPO₄ M/10	KH₂PO₄ M/7	HCl M/10			0.4	0.28	0.2	0.14	0.1	0.07	0.05		Gravimetric	Molar
						Proportion of motile organisms after 12 hours at 25°C.									
0.02	0.38			8.42	1:10,000	0	20	72	>				0.24	0.34	0.5
0.01	0.39			8.10	1:10,000		0	54	>				0.21	0.39	0.56
		4:1 mixture		7.32	1:10,000						10	57	0.055	1.5	2.1
		2:1 mixture		6.97	1:80,000		21	88					0.25	2.7	3.8
		1:1 mixture		6.64	1:80,000			4	56				0.153	4.3	6.1
		1:2 mixture		6.48	1:80,000				3	66			0.113	5.9	8.4
		1:4 mixture		6.18	1:80,000					5	69		0.085	7.8+	11.2
		1:8 mixture		6.02	1:80,000						4	79	0.059	11.2	16.0
		0.4		5.77	1:320,000		15	66					0.165	16	22.8
		0.385	0.015	5.65	1:320,000			35	>				0.133	20	28.6
Buffer mixture No. 3 with x cc. of 1:2,000,000 phenyl arsenoxide.				7.32	1:2,000,000			18	62				0.165	100	100

ᵃ Gravimetric activity = $\dfrac{\text{cc. of unsubstituted phenyl arsenoxide necessary to immobilize 55\% of organisms}}{\text{cc. of unknown arsenical necessary to immobilize 55\% of organisms}} \times \dfrac{\text{Concn. of phenyl arsenoxide}}{\text{Concn. of unknown arsenical}} \times 100.$

emulsifying acute testicular syphilomata of rabbits; and T. equiperdum, harvested from the blood of white rats or mice, was the organism used for the trypanocidal assays.

In determining the effect of pH on the activity of the compounds, varying isotonic mixtures of Na_2HPO_4 and KH_2PO_4 were added to a final concentration of M/80–M/120 in the drug-organism mixture, as indicated in table 2. The pH of the final mixture was determined with the glass electrode (Beckman pH meter); and no significant change in pH occurred in the course of the two to three hours for which the organisms were left in contact with the drug, prior to the determination of the proportion of surviving (motile) organisms.

The method used to demonstrate the binding of arsenicals by trypanosomal suspensions (6), and the analytical procedure (7), have been described in previous communications from this laboratory.
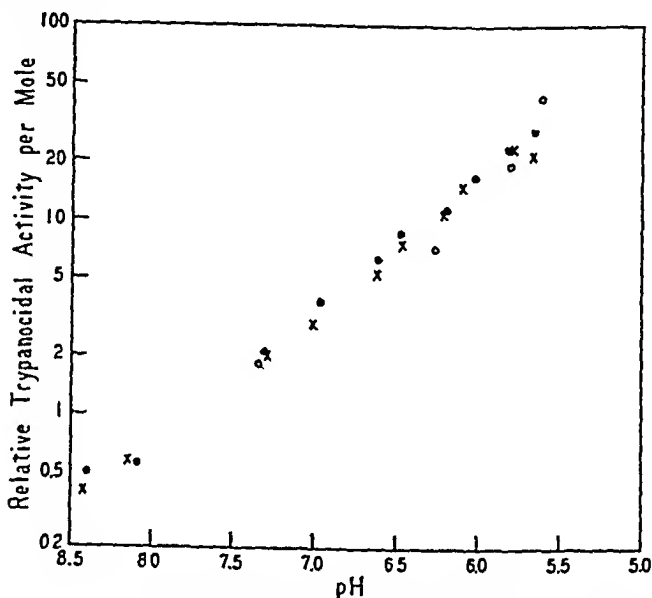
FIG. 1. THE EFFECT OF pH ON THE TRYPANOCIDAL ACTION (T. EQUIPERDUM)
OF p-(CH₂)₂COOH PHENYL ARSENOXIDE

The points in the figure refer to three similar experiments, carried out at different times. The filled circles refer to the experiment summarized in table 2. The curve in fig 2 (●—●) is a composite of the three experiments.

EXPERIMENTAL. *The effect of pH on the trypanocidal and spirocheticidal activity of acid-substituted phenyl arsenoxides.* A single experiment illustrating the effect of pH on the trypanocidal activity of an acid-substituted phenyl arsenoxide (p-(CH₂)₂COOH) is summarized in table 2. The results of three such experiments with the same compound are graphically summarized in fig. 1. As is there shown, there was a progressive increase in the trypanocidal activity of the compound as the pH was decreased from 8.4 to 5.65, the activity at pH 5.65 being seventy-five times greater than that at pH 8.4. The curve for this compound in fig. 2 is a composite of the experimental data of fig. 1, obtained by averaging the interpolated trypanocidal activities at a series of pH values (8.5, 8.25, 8.0, 7.5, 7.0, 6.5, and 6.0).

Similar experiments were carried out with a total of eleven acid-substituted compounds. The results for ten of those compounds are summarized in fig. 2, the curve for the 3-NO₂-4-COOH phenyl arsenoxide having been omitted for clarity (cf. table 3). Each curve in that figure is a composite of two to five experiments resembling those summarized in fig. 1. The striking and regular effect of pH on the trypanocidal activity of most of the compounds is evident from the
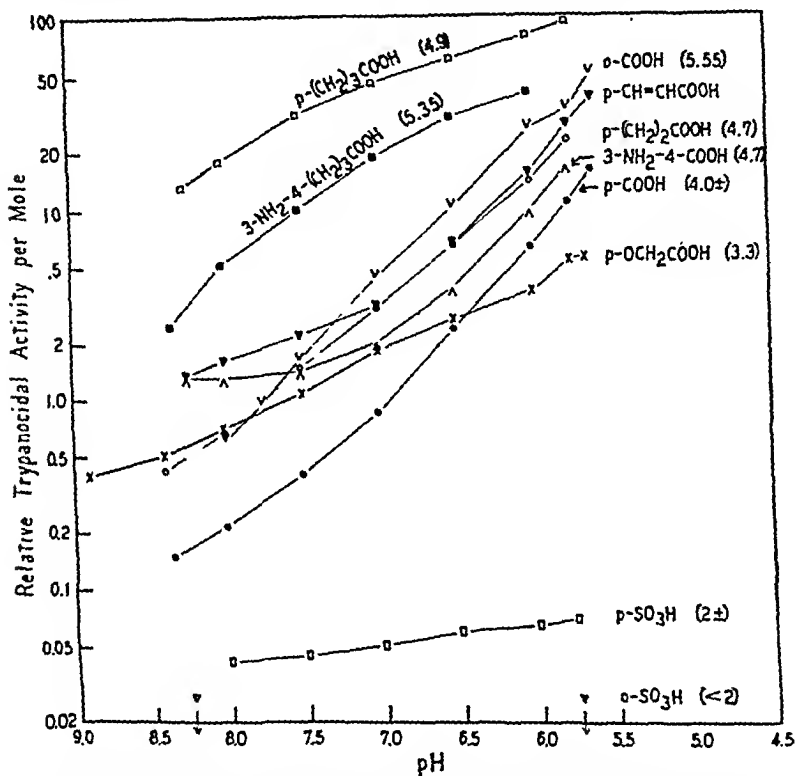
FIG. 2. THE EFFECT OF pH ON THE TRYPANOCIDAL ACTION OF A SERIES OF ACID-SUBSTITUTED PHENYL ARSENOXIDES

Each curve in the figure is an average of 3 to 5 experiments resembling those of table 2 and figure 1. The numbers in (□) are the pK values of the substituent acidic groups.

figure. The apparent exceptions provided by the p-SO₃Na and o-SO₃Na compounds are particularly to be noted, and are discussed in a following paragraph.

Experiments with S. pallida as the test organism instead of T. equiperdum, using the o-COOH and p-(CH₂)₃COOH phenyl arsenoxides, are summarized in fig. 3. The results were qualitatively similar to those obtained with trypanosomes, in that there was a progressive increase in activity as the pH was shifted toward the acid side.

The relationship between the trypanocidal activity of acid-substituted phenyl

arsenoxides and the pK of the acidic substituents. Several distinct lines of evidence indicated that the striking effect of pH on the trypanocidal and spirocheticidal activity of acid-substituted phenyl arsenoxides was related to the ionization of the acidic group.

TABLE 3

The correlation between the observed trypanocidol activity of acid-substituted phenyl arsenoxides at pH 6 0, and that calculated from the pK of the acidic substituents

1	2	3	4	5	6	7	8	9	10
		OBSERVED MOLAR ACTIVITY OF COMPOUND AT pH 6 0	CALCULATED ACTIVITY DUE TO IONIZED FRACTION				CALCULATED ACTIVITY AT pH 6 0 DUE TO FREE ACID[d] (= 6)	CALCULATED ACTIVITY OF COMPOUND (7 + 8)	RATIO OF OBSERVED CALCULATED ACTIVITY AT pH 6 0 (3 ÷ 9)
COMPOUND	pK[a]		Molar activity of compound (ion[b] at pH 8 0)	Percentage of compound present at pH 6 0 as		Calculated activity at pH 6 0 due to salt[c] (4 × 5)			
				Ionized salt	Undissociated free acid				
o-SO₃Na	<2*	<0.015	<0.015	>99 99	<0.01	<0.015	<0.015	<0.015	
p-SO₃Na	2±	0.066	0.042	99.99	0.01	0 042	0.01	0.052	1.3
3-NO₂-4-COOH	2.6	33	17.0	99.96	0.04	17.0	0.04	17	1.9
p-OCH₂COOH	3 3	2.7	0.33	99 8	0.2	0.33	0.2	0.53	5.1
p-COOH	4.0	3.0	0.04	99	1	0 04	1.0	1.04	2.9
3-NH₂-4-COOH	4.7	5 7	0 6	95	5	0 57	5.0	5.6	1.0
p-(CH₂)₂COOH	4.9	42	6.9	92.7	7 3	6 4	7.3	13.7	3.1
p-(CH₂)₄COOH	5.23*	31	3 7	85 4	14.6	3 2	14.6	17.8	1.74
3-NH₂-4-(CH₂)₂ COOH	5 34*	33	3 0	82	18	2.5	18	20.5	1.6
p-(CH₂)₃COOH	5.35±*	40	1 5	81 7	18.3	1 2	18 3	19.5	2.05
o-COOH	5.55	16	0.52	74	26	0.39	26 0	26	0.61

* • pK values indicated with asterisk not previously reported. cf. (1).

[b] Activity of compound at pH 8 0 taken as activity of ionized salt. As is discussed in the text, this maximum value is in error to the extent that the compound is not completely dissociated at that pH, and to the extent also that the trypanosomes combine with the traces of free acid. All trypanocidal activities in this and following columns are referred to that of phenyl arsenoxide as 100

[c] Molar activity of salt × percentage of ionized salt at pH 6 0 (column 4).

[d] Assuming the free acid to be as active as the undissociated phenyl arsenoxide. Since the latter is assigned an arbitrary molar activity of 100, the percentage of compound present as free acid at pH 6.0 is the molar activity due to free acid

As is shown in fig. 4, the activity of the unsubstituted phenyl arsenoxide was largely independent of pH over the range pH 5.7 to 8.7. This was true also of the 3-NH₂-4-OH phenyl arsenoxide, in which neither of the substituents was strongly acidic. Most significant of all, when an acidic group was blocked, as by amide formation, the trypanocidal activity was no longer dependent on pH, but remained essentially constant in the range pH 5.7 to 8.7[1] (cf. the p-CONH₂, p-SO₂NH₂ and p-CONHCH₂CONH₂ compounds in fig. 4).

[1] At pH values of 8 and more, there was a distortion in the pH-activity curves of non-acidic phenyl arsenoxides (cf. fig. 4), which presumably also affects those with acidic substituents, and which makes the curves in that range difficult to interpret. This distortion in the pH-activity curve may perhaps be referable to the beginning ionization of the —AsO group considered as an acid (1).
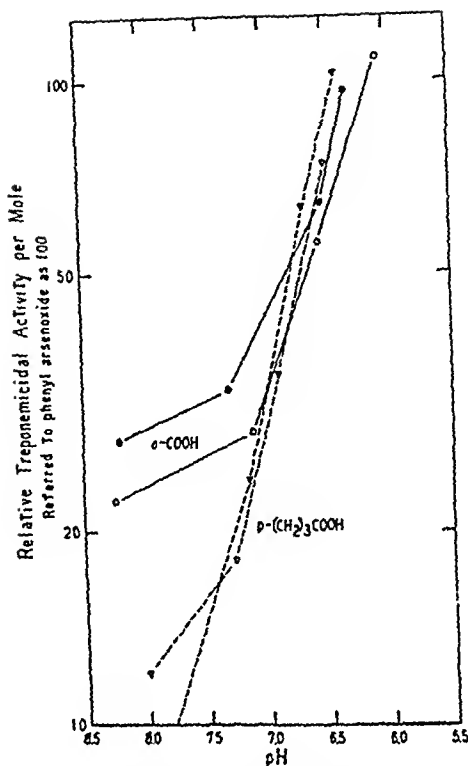
FIG. 3. THE EFFECT OF pH OF THE SPIROCHETICIDAL ACTION OF o-COOH—
AND p-(CH₂)₃COOH— PHENYL ARSENOXIDES

The experiments resemble that illustrated in table 1, but were carried out with a suspension of pathogenic S. pallida (Nichols strain) as the test organism instead of T. equiperdum. The method of preparing the suspension has been described elsewhere[5]. Each curve in the figure refers to an individual experiment.
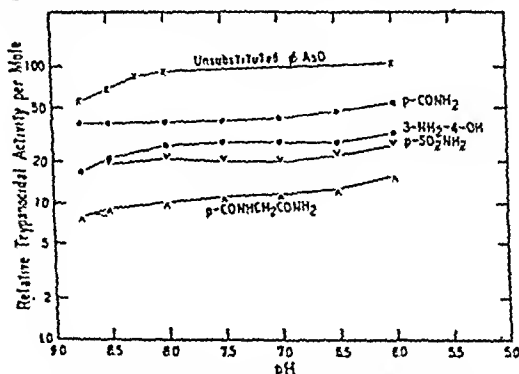
FIG. 4. THE (NEGLIGIBLE) EFFECT OF pH ON THE TRYPANOCIDAL ACTION OF
PHENYL ARSENOXIDES LACKING ACIDIC SUBSTITUENTS

Including compounds in which an acidic substituent had been blocked by amide formation.

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The striking effect of pH on the activity of acid-substituted phenyl arsenoxides was therefore not due to an altered susceptibility of the organisms, or to the varying activity of the —AsO group as such. The acidic function of the substituent group was clearly implicated. The most obvious effect on these compounds of an increasing hydrogen ion concentration consists in the formation of the undissociated free acid from the ionized salt[2], e.g.,

$$OAs\langle\bigcirc\rangle COO- \underset{OH^-}{\overset{H^+}{\rightleftarrows}} OAs\langle\bigcirc\rangle COOH.$$

The inference from the data of the preceding section is that the free acid is many times more active than the charged ion. If that inference is correct, there should be a close correlation between the trypanocidal activity of an acid-substituted compound at a given pH, and the pK of the acidic substituent. The higher that pK, and thus, the higher the proportion of the free acid at a given pH, the greater should be the activity of the compound. Conversely, the lower the pK (i.e., the stronger the acidic group), the lower the pH at which there is a significant proportion of undissociated molecules, and the lower should be the pH at which one first observes an increase in trypanocidal activity.

These relationships between pK and trypanocidal activity were confirmed experimentally, and are clearly indicated in the right hand portion of fig. 2. *With a strongly acidic compound such as the p-SO₃H phenyl arsenoxide, in which* the pK of the acidic group was approximately 2.0, 99.9 per cent of the compound would be present as the (inactive) ionized salt at pH 6.0. Less than 1/10,000th would be present as the (active) undissociated free acid; and the relative trypanocidal activity at that pH was correspondingly low (0.06, or 1/1700th that of the unsubstituted phenyl arsenoxide). As anticipated, there was no demonstrable change in trypanocidal activity in the range pH 8.5 to 5.5. At the other extreme, in the case of the o-COOH compound, with a pK of 5.55, approximately 25 per cent would be present as the undissociated free acid at pH 6.0; and the relative trypanocidal activity of that compound at that pH was correspondingly high (19). Acidic groups with intermediate pK values usually had correspondingly intermediate activities (cf. fig. 2). The exceptions provided by the p-(CH₂)₃-COOH and 3-NH₂-4-(CH₂)₃COOH phenyl arsenoxides were in part due to the unusually high trypanocidal activity of their ionized salts. This, adding on to the activity due to the undissociated molecule, contributed to the unexpectedly high activity of those compounds at pH 6.0[3].

[2] $pH = pK' + \log \dfrac{[salt]}{[acid]}$ $\qquad \log \dfrac{[salt]}{[acid]} = pH - pK'$

[3] As shown in the left hand portion of fig. 2, and as was to have been anticipated, the clear correlation between the pK of the substituent group and the trypanocidal activity of acid-substituted phenyl arsenoxides at pH 6.0 was obscured at more alkaline reactions. At pH 6.0, where a significant proportion was present as the undissociated free acid, the observed activity was preponderately due to that fraction, and was therefore quantitatively related to the pK of the compound. Thus, if the pK of an acid-substituted phenyl arsenoxide were 5.0, at pH 6.0 approximately 9 per cent of the compound would be present as the undissociated free acid. If the latter is assumed to have an activity 100 times greater than

The data of fig. 2 were, however, rendered of only qualitative significance with the finding that the trypanosomes selectively bound the undissociated free acid, and had but little affinity for the relatively inactive ion (cf. following section). Since the solutions were strongly buffered, and the ratio of salt:acid thereby stabilized, the removal of the free acid from solution by the organisms necessarily caused more of the compound to go over into that form, making more available for the trypanosomes. Inasmuch as the trypanocidal activity of an arsenical is determined by the amount bound by the organisms (6), the trypanocidal activity of these acid-substituted compounds was regularly greater than the pK of the acid would have implied. In a sense, the organisms promoted their own death by binding the active form of the compound, and thus shifting the equilibrium so as to form more of it.

It follows from these considerations that a) the heavier the trypanosomal suspension, the greater should be the apparent activity of an acid-substituted phenyl arsenoxide relative to that of a non-acidic compound simultaneously tested; and conversely, b) the fewer organisms used, the smaller would be the error introduced by their selective combination with the free acid, and the more closely would the observed trypanocidal activity correspond to the proportions of free acid and ion originally present in the solution.

The first conclusion was confirmed by the experiments summarized in fig. 5. With two representative compounds, the apparent trypanocidal activity throughout the pH range 5.5 to 8.4 increased with the number of organisms. A six-fold increase in that number resulted in a two-to four-fold increase in trypanocidal activity, referred to that of the unsubstituted phenyl arsenoxide simultaneously tested as 100.

Were it not for the large error introduced by this selective affinity of the organisms for the free acid, it would be theoretically possible to calculate the trypanocidal activity of an acid-substituted phenyl arsenoxide at a given pH from the proportion of ionized and non-ionized fractions, determined by the pK of the compound. The activity at pH 8.0 could be taken as an approximation of the molar activity of the ion[4]. The molar activity of the free acid is, as a

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that of the ion (the usual order of magnitude; cf. following paragraphs in text), the activity at pH 6.0 would be $(0.09 \times 100) + (0.91 \times 1) = 9.91$, of which only 0.91 would derive from the ion; and even considerable variation in the activity of the ion would have little effect on the total observed activity. On the other hand, at pH 8.0, only 1/1000th of the compound would be undissociated, and the activity would be $(0.001 \times 100) + (0.999 \times 1) = 1.1$, of which 0.999 would derive from the ion. The varying and slight molar activity of the ions of the various acid-substituted compounds would nevertheless suffice to obscure the quantitative relationship between trypanocidal activity and the pK of the acidic substituent. This is evident in the left hand portion of fig. 2, and is further borne out by the data of table 3. These relationships probably explain, at least in part, the previously noted poor correlation between the spirocheticidal action of acid-substituted phenyl arsenoxides at pH 7.0–7.4, and the pK of the acidic substituents (1).

[4] This approximation would be in error to the degree that the compound is not completely dissociated at that pH. This otherwise negligible discrepancy would be exaggerated by the fact that the organisms bind the traces of free acid present at the pH, and thus cause the continuing formation of amounts in excess of that calculated from the pK of the acidic substituent.

The striking effect of pH on the activity of acid-substituted phenyl arsenoxides was therefore not due to an altered susceptibility of the organisms, or to the varying activity of the —AsO group as such. The acidic function of the substituent group was clearly implicated. The most obvious effect on these compounds of an increasing hydrogen ion concentration consists in the formation of the undissociated free acid from the ionized salt[2], e.g.,

$$OAs\langle\bigcirc\rangle COO- \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} OAs\langle\bigcirc\rangle COOH.$$

The inference from the data of the preceding section is that the free acid is many times more active than the charged ion. If that inference is correct, there should be a close correlation between the trypanocidal activity of an acid-substituted compound at a given pH, and the pK of the acidic substituent: The higher that pK, and thus, the higher the proportion of the free acid at a given pH, the greater should be the activity of the compound. Conversely, the lower the pK (i.e., the stronger the acidic group), the lower the pH at which there is a significant proportion of undissociated molecules, and the lower should be the pH at which one first observes an increase in trypanocidal activity.

These relationships between pK and trypanocidal activity were confirmed experimentally, and are clearly indicated in the right hand portion of fig. 2. With a strongly acidic compound such as the p-SO₃H phenyl arsenoxide, in which the pK of the acidic group was approximately 2.0, 99.9 per cent of the compound would be present as the (inactive) ionized salt at pH 6.0. Less than 1/10,000th would be present as the (active) undissociated free acid; and the relative trypanocidal activity at that pH was correspondingly low (0.06, or 1/1700th that of the unsubstituted phenyl arsenoxide). As anticipated, there was no demonstrable change in trypanocidal activity in the range pH 8.5 to 5.5. At the other extreme, in the case of the o-COOH compound, with a pK of 5.55, approximately 25 per cent would be present as the undissociated free acid at pH 6.0; and the relative trypanocidal activity of that compound at that pH was correspondingly high (19). Acidic groups with intermediate pK values usually had correspondingly intermediate activities (cf. fig. 2). The exceptions provided by the p-(CH₂)₃-COOH and 3-NH₂-4-(CH₂)₃COOH phenyl arsenoxides were in part due to the unusually high trypanocidal activity of their ionized salts. This, adding on to the activity due to the undissociated molecule, contributed to the unexpectedly high activity of those compounds at pH 6.0[3].

[2] $pH = pK' + \log \dfrac{[salt]}{[acid]} \qquad \log \dfrac{[salt]}{[acid]} = pH - pK'$

[3] As shown in the left hand portion of fig. 2, and as was to have been anticipated, the clear correlation between the pK of the substituent group and the trypanocidal activity of acid-substituted phenyl arsenoxides at pH 6 0 was obscured at more alkaline reactions. At pH 6 0, where a significant proportion was present as the undissociated free acid, the observed activity was preponderately due to that fraction, and was therefore quantitatively related to the pK of the compound. Thus, if the pK of an acid-substituted phenyl arsenoxide were 5 0, at pH 6 0 approximately 9 per cent of the compound would be present as the undissociated free acid. If the latter is assumed to have an activity 100 times greater than

phenyl arsenoxides were tested at a series of pH values by the technic of table 2, but with a suspension containing only 5×10^5 organisms per cc. The experimentally determined molar trypanocidal activities of these compounds at pH 6.0 and 8.0 are listed in columns 2 and 3 of table 3. Those activities were regularly less than had previously been obtained with much heavier suspension (cf. figs. 2 and 5). The theoretical activities at pH 6.0, listed in the next to last column of that table, were calculated on the assumptions a) that the activity at pH 8.0 was that of the free ion[5], b) that the activity of the free acid was, like
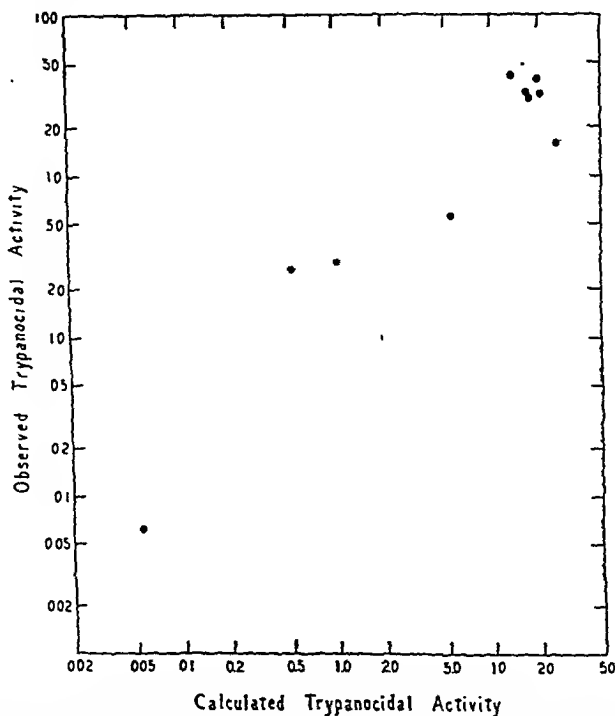
FIG. 6. THE CORRELATION BETWEEN THE OBSERVED TRYPANOCIDAL ACTIVITY OF PHENYL ARSENOXIDES AT pH 6.0 AND THE ACTIVITIES CALCULATED FROM THE pK OF THE ACIDIC GROUP (CF. TABLE 3)

that of the —CH$_3$, —Cl or —NO$_2$ substituted compounds, essentially the same as that of phenyl arsenoxide[5], and c) that the trypanosomal suspension used did not bind significant amounts of free acid, so that the proportions of free acid and ion at pH 6.0 were determined by the pK of the acid substituents, as indicated in columns 5 and 6 of table 3.

The degree to which the calculated and observed activities agreed is evident on comparing columns 3, 9, and 10 of table 3, and is graphically shown in fig. 6. With only one exception, the observed activity was regularly greater than the

maximum, that of the unsubstituted phenyl arsenoxide[5]. The relative proportions of ion and free acid at e.g. pH 6.0 could be calculated by the pK of the compound, but would be in error to the extent that the free acid was bound by the trypanosomes, as previously discussed.
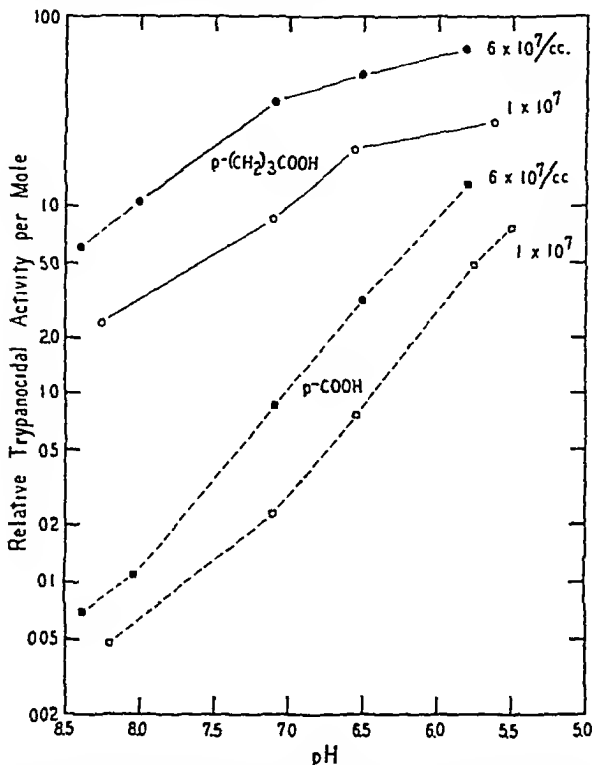
FIG. 5. THE EFFECT OF THE NUMBER OF ORGANISMS ON THE APPARENT TRYPANOCIDAL ACTIVITY OF TWO ACID-SUBSTITUTED PHENYL ARSENOXIDES

A suspension of trypanosomes containing the indicated number of organisms per cc. was added to equal volumes of arsenical and of buffer, after the technic of table 2. The final concentration of trypanosomes in the reacting mixture was therefore one-third of that indicated in the figure. The trypanocidal activities are in each instance referred to that of phenyl arsenoxide simultaneously tested, with the same suspension, as 100.

In the experiments summarized in table 3, an attempt was made to minimize that error by using a thin suspension of trypanosomes. Twelve representative

[5] Of more than 150 substituted phenyl arsenoxides tested to date with respect to spirocheticidal and trypanocidal activity, none has been significantly more active than the parent unsubstituted compound. Some "indifferent" substituents had no effect, while most substituent groups inhibited activity to a varying degree. It is therefore reasonable to assume that the molar activity of an undissociated acid-substituted phenyl arsenoxide is, as a maximum, that of the unsubstituted compound.

compounds the activity of which increased with acidity due to the formation of free acid from the ion, there would be a corresponding increase in the amount of arsenical bound by the organisms. As shown in fig. 7, this was confirmed with the p-$(CH_2)_3COOH$, 3-NH_2-4-$(CH_2)_3COOH$ and p-COOH phenyl arsenoxides. A single experiment is described in detail in table 4.

In the case of those compounds with more strongly acidic substituents (e.g., the p-OCH_2COOH and 3-NO_2NO_2-4-COOH phenyl arsenoxides, with pK values of 3.3 and 2.6 respectively), the increased binding, like the increased activity, did not become apparent until the solution had been made sufficiently acid to produce

TABLE 4

Illustrating the effect of pH on the binding of an acid-substituted phenyl arsenoxide (3-NH_2-4-$(CH_2)_3COOH$) by T. equiperdum

The organisms sedimented from rat blood were resuspended in 30 per cent rabbit serum containing 0.2 per cent added glucose, to a concentration of 500 x 10^6 organisms per cc. Five cc. were rapidly added to a mixture of 4 cc. of isotonic phosphate buffer, and 1 cc. of arsenical solution containing 16.7 γ of arsenic. The contents were immediately mixed, and the tube sharply centrifuged after ten minutes at room temperature. The volume of the sedimented organisms averaged 0.12 cc. at pH 7.4. Although there was often a slight increase in that volume with increasing acidity, averaging 0.02–0.03 cc. at pH 5.5, that change was ignored in calculating the ratio of arsenic concentrations in the trypanosomes and in the surrounding fluid.

EXPT. NO.	BUFFER USED				pH	ARSENIC CONTENT, MICROGRAMS[a] OF		% OF TOTAL ARSENIC IN TRYPANOSOMES	RATIO OF ARSENIC CONCN. ORGANISMS / ARSENIC CONCN. FLUID
	NaOH N/10	Na₂HPO₄ M/10	KH₂PO₄ M/7	HCl N/10		Trypanosomes	Supernatant		
2	1:9				8.08	1.65	15.5	9.6	8.8
1		100			7.76	1.96	15.5	11.2	10.4
2		4:1			7.22	3.6	11.5	24	26
1		2:1			6.88	4.65	13.5	26	28
2		1:4			6.04	5.7	12.0	32	39
1		1:8			5.88	6.7	9.7	41	57
2			100		5.39	10	7.7	57	107
1				19:1	5.2	10.0	7.6	57	109

[a] Actual content of arsenic, and not concentration.

significant if slight amounts of the free acid (cf. fig. 8). (The relatively high degree to which the 3-NO_2-4-COOH was bound at pH 9.0–6.5 reflects the anomalous high activity of the ion of this compound). With the most strongly acidic compound tested, the o-SO_3Na compound, with a pK value of less than 2, less than 1/3000th of the compound would be present as the free acid even at the lowest pH tested, 5.5; and there was no demonstrable difference in either activity or binding between pH 5.4 and 9 (cf. figs. 2 and 8). Finally, in the case of the unsubstituted phenyl arsenoxide, or of the p-$CONH_2$ compound, in which an acidic group had been blocked by amide formation, increasing acidity did not result in an increased binding of arsenic by the trypanosomes, corresponding to

theoretical. This suggests that even the thin suspension used in these experiments bound enough of the free acid to enhance the apparent trypanocidal activity of the compound significantly. Nevertheless, the correlation between the observed and theoretical values was generally ·satisfactory, and permits the conclusion that the relative trypanocidal activity of an acid-substituted phenyl arsenoxide is determined by, and is roughly predictable from, a) the pH of the solution, b) the pK of the acid substituent, and c) the trypanocidal activity of
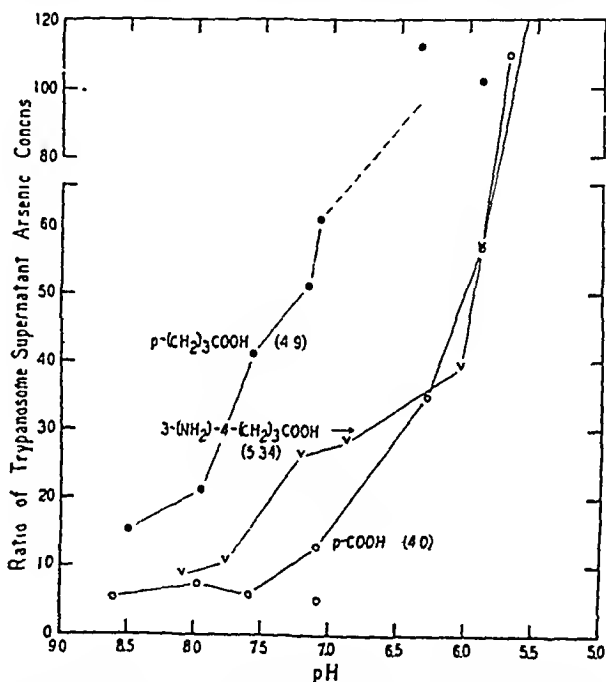
FIG. 7. THE EFFECT OF pH ON THE AFFINITY FOR TRYPANOSOMES OF THREE ACID-SUBSTITUTED PHENYL ARSENOXIDES WITH RELATIVELY WEAK ACIDIC GROUPS

Summary of three experiments resembling that of table 4. The numbers after the substituent groups in the figure are the pK of the acidic groups. The marked effect of pH on the affinity of the arsenical for trypanosomes parallels the similar effect on their trypanocidal activity (cf. fig. 2).

the ion. The higher the pK, and the lower the pH, the greater will be the observed activity of the compound.

The effect of pH on the binding of acid-substituted phenyl arsenoxides by trypanosomes. It has been shown in a preceding paper (6), that the varying trypanocidal activity of a series of phenyl arsenoxides reflects the varying degree to which these arsenoxides are bound by the trypanosomes. On the basis of the preceding discussion, it was therefore to have been anticipated that in the case of those

groups, which remain as ions at the lowest pH tested, are correspondingly unaffected with respect to both activity and combining affinity.

DISCUSSION. There is a considerable body of evidence that arsenicals combine with —SH groups of living cells. Voegtlin, Dyer and Leonard (8) tentatively identified those —SH groups with glutathione, and ascribed the parasiticidal action of arsenicals to that combination. Rosenthal and Voegtlin (9) and Schmitt and Skow (10) later suggested that other —SH groups may be concerned, in particular the "fixed" —SH groups of tissue protein. More recently, Barron and Singer (11) have shown that arsenicals combine reversibly with the —SH groups in a series of enzyme proteins, the enzymatic function of which is thereby suppressed. These observations provide a simple and rational explanation for the parasiticidal action of phenyl arsenoxides.

The present demonstration that undissociated acid-substituted phenyl arsenoxides are so much more strongly bound by trypanosomes than their ionized salts may reflect a difference between the combining affinities of the free acids and their ions for the reactive cellular —SH groups. However, no such difference between acidic and non-acidic compounds was apparent when phenyl arsenoxides were reacted in vitro with, e.g., cysteine to form thioarsenites, nor was there any demonstrable difference in the degree to which the formed thioarsenites hydrolyzed at pH 7.4.

Conceivably, also, the electrostatic repulsion between the negatively charged ion and the negatively charged trypanosome might prevent their combination. The paradoxically high activity of the $3\text{-}NO_2\text{-}4\text{-}COOH$, $p\text{-}(CH_2)_2COOH$, $p\text{-}(CH_2)_4COOH$ and $3\text{-}NH_2\text{-}4\text{-}(CH_2)_3COOH$ phenyl arsenoxides at pH 7.4 (cf. table 1) seems to negate that possibility. Like the other acid-substituted compounds, these compounds as free acids were more strongly bound and correspondingly more active than they were as ions. Unlike the other acid-substituted compounds, however, their ions also were significantly bound by the organisms, and possessed a high degree of activity (cf. table 3 and figs. 7 and 8). Clearly, the ionic charge as such is not the sole factor responsible for the generally low affinity of ionized phenyl arsenoxides for trypanosomes.

The most reasonable explanation for the relative inactivity of the ionized salts of acid-substituted phenyl arsenoxides is that they may be unable to pass through the cell membrane into the interior of the cell. Highly active phenyl arsenoxides are bound by trypanosomes in amounts difficult to explain on the basis of combination solely with reactive groups on the surface of the cell[6]. Those compounds

[6] Reiner, Leonard and Chao (24) have calculated that the amount of arsenic bound by trypanosomes in their experiments (0 1 microequivalent per 10^{10} organisms, or 6×10^6 molecules per trypanosome) is consistent with its combination with —SH groups on the surface of the organisms In our own experiments, however, using more active arsenicals, twenty times as much arsenic has been bound (up to 30 micrograms by 2×10^9 organisms, or $1\ 2 \times 10^8$ molecules per trypanosome, with a surface area of approximately 10^{-6} sq cm.). If these were all on the surface of the organism, the mean distance between their points of attachment would be only 10^{-7} cm This would imply that on the order of every fifth atom on the surface of the trypanosome is an —SH sulfur (or some other group equally reactive with arsenical), far exceeding the analytical values for free —SH groups in proteins

the fact that the activity of the compound was also not significantly affected (cf. fig. 3).

It follows from these considerations that the trypanocidal activity of an acid-substituted phenyl arsenoxide in the pH range 5.5–9.0 is a function of the amount of arsenical bound. The varying activity of the compound within that pH range reflects the varying proportions of the ionized salt, with a relatively slight
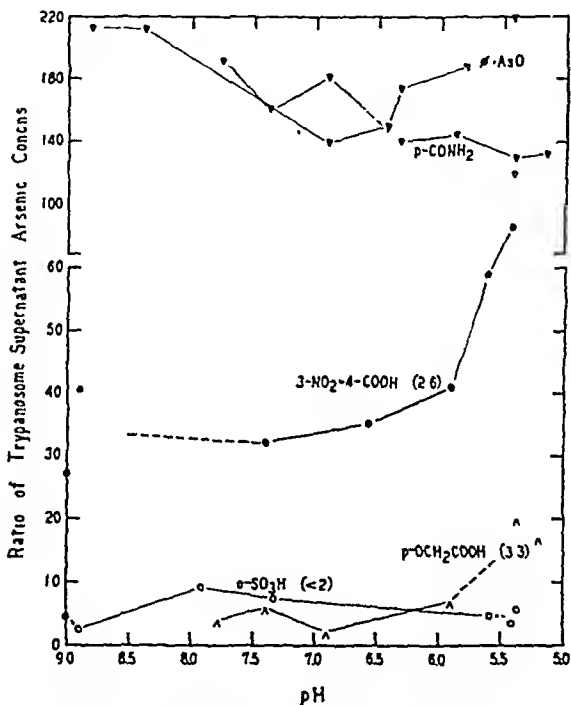
FIG. 8. THE EFFECT OF pH ON THE AFFINITY OF TRYPANOSOMES FOR PHENYL ARSENOXIDES WITH STRONGLY ACIDIC OR NON-ACIDIC SUBSTITUENTS

Summary of five experiments resembling that of table 4. The numbers after the substituent groups in the figure are the pK values of the acidic groups. a) The failure of pH to affect the affinity for trypanosomes of non-acidic and b) the fact that the stronger the acidic group, the more acid the reaction at which the arsenicals develop an increased affinity for trypanosomes, again parallel the similar effects of pH on their trypanocidal activity (cf. figs. 2 and 3).

affinity for the trypanosome and a correspondingly slight activity, and of the undissociated free acid, which is strongly bound by the trypanosome and is correspondingly active. In a series of acid-substituted phenyl arsenoxides, those with the highest pK values, most readily transformed to the free acid on acidification, are the first to show an increased activity and an increased affinity for the trypanosomes as the reacting mixture is acidified; while those with strongly acidic

with alkalinization in the pH range 6.5–8.0, ascribed by Goljachowski (21) to the formation of the undissociated free base, which was bound by the heart tissue. Brucine was found to diffuse into Nitella only as the free base (Irwin (22)), and accumulated within the cell to the degree that it was there transformed to the ionized salt, permitting the diffusion of more of the free base. This and similar "trap mechanisms" leading to the intracellular accumulation of freely diffusing molecules have been described by many workers. Finally, a variety of cells (epidermal cells of leaves of Rheo discolor, cilia of Tradescentia virginica, and root cells of Lemna minor) were found by Poijäri (23) to be permeable to undissociated weak bases, but not to their cations.

The present demonstration that the ions of acid-substituted phenyl arsenoxides are bound by trypanosomes to a far less degree than the corresponding free acids or non-acidic arsenoxides, is thus a phenomenon encountered with a wide variety of cells and compounds. Various explanations have been offered for that phenomenon, the discussion of which is not within the scope of the present paper. The distinguishing feature of the trypanosome-arsenical system here studied lies in the speed, and particularly in the degree to which active, non-ionized arsenicals may be concentrated by the trypanosomes. Within ten minutes, the arsenic concentration in the organisms may attain a level several hundred times greater than that of the surrounding fluid. This reflects a rapid diffusion of the arsenical into the organisms, followed by its firm combination with cellular constituents. The arsenical is thereby effectively removed from the diffusion equilibrium, permitting its continued diffusion into the cell. In comparison with the effect of this combination in permitting the accumulation of arsenic in the trypanosomes, the partial conversion of some of the acid to the ionized form within the cell is a quantitatively negligible factor.

It is interesting to note that even the most inactive of the ionized phenyl arsenoxides here studied were nevertheless bound by the trypanosomes to a certain degree, attaining a concentration in the organisms several times that in the surrounding fluid (cf. table 1 and fig. 8) without exerting any apparent deleterious effect on the organisms. This degree of concentration may perhaps be due to the binding of arsenoxides by —SH groups on the surface of the organism. In such case, it would follow that these surface groups are not vital to the cell, either because they are qualitatively different from those in its interior, or because quantitatively not enough —SH groupings are thereby blocked to affect the viability of the organism.

SUMMARY

1. The trypanocidal and spirocheticidal activity of most of the acid-substituted phenyl arsenoxides here studied increased strikingly with increasing hydrogen ion concentration in the pH range 5.5–9.0.

2. This was due to the fact that the ionized salts of these compounds were only a fraction as active as the undissociated free acids, which had a uniform molar activity approximating that of unsubstituted phenyl arsenoxide.

3. The relative activity of an acid-substituted phenyl arsenoxide at a given pH

apparently combine with —SH or other groupings within the cell; and it is probable that the salts of acid-substituted arsenoxides are not bound, and thus inactive, because of their relative inability to pass through the cell membrane.

That the ions of weak acids (or bases) pass through cell membranes with greater difficulty than the corresponding undissociated molecules is a phenomenon which has been described by many workers and for a wide variety of cells. Thus, Vermast (12) found that the bactericidal action of benzoic and salicylic acids increased with increasing hydrogen ion concentration. Labes (13), working with tadpoles and S. aureus, made a similar observation in the case of butyric, caproic, and benzoic acids, and attributed the effect to the more rapid penetration of the cells by the undissociated acid molecules. The cilia of Mytilus edulis were far more strongly inhibited by weak acids (acetic and butyric) than they were by HCl at the same pH, and NH_3 was more active than NaOH (Gray (14)), in both cases due to the ability of the undissociated weak alkalis and acids to penetrate into the cell. Similarly, starfish eggs were penetrated by undissociated CO_2 and NH_4OH, but not by their ions (Jacobs (15)); a plant cell, Valonia macrophysa, was penetrated by CO_2 and H_2S only as undissociated molecules, and not as HS^-, HCO_3^-, or CO_3^- ions (Osterhout et alii (16)); and in the activation of starfish eggs (Asterias forbesii) by a series of acids, only the undissociated molecules were found to penetrate the cells, the actual activation by H^+ being due to their intracellular ionization (Lillie (17)). A series of substituted phenols penetrated the eggs of Arbacia punctulata only as undissociated molecules, and the degree to which these compounds inhibited cell division was determined by the concentration of those molecules in the external medium, and thus, in the cell itself (Krahl and Clowes (18)).

Of particular interest is the fact that, in contradistinction to the foregoing observations with weak acids, and consistent with the general thesis that cells are penetrated by undissociated molecules more readily than by their ions, the activity of weak bases is increased by alkalinization rather than acidification. Prowazek (19) made that observation in studying the toxicity of the alkaloids atropine, quinine, and strychnine for Colpidium, and similar observations were made by Crane (20), working with Paramecium caudatum[7]. The effect of quinine, (dissociation constant 2.2×10^{-7}) on the isolated frog heart also increased

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so far studied in that respect. An even greater disparity between the amount of arsenic bound and the known or probable density of —SH groups on the surface of the cell has been noted in the case of red blood cells (Magnuson, Eagle and Watson, unpublished data).

[7] The apparent exception provided by caffeine, the activity of which was independent of pH in the range 5.9–8.7, was due to the fact that, with a dissociation constant of 1.4×10^{-10}, it remains almost completely ionized in the pH range studied; while in the case of the other compounds tested by Crane, with dissociation constants of 1–17×10^{-7}, the degree of dissociation varied materially within that pH range, with a corresponding change in activity. An analagous situation has been described in the present paper in the case of the strongly acidic o-SO_2Na and p-SO_2Na compounds, with dissociation constants greater than 10^{-2}. These compounds would remain almost completely ionized in the pH range 5.0–9.0, and they remained correspondingly inactive throughout that range, in contrast to the other acids studied, with pK values of 3.3–5.5.

THE SYMPATHOMIMETIC VASODILATING ACTION OF THE ALIPHATIC AMINES

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Three aliphatic amines have been found to have significant sympathomimetic activity. These are, 2-methylamino-iso-octene (Oetin), 2-methylamino-heptane (EA-1) and 2-amino-heptane (Tuamine). These compounds on intravenous administration to animals produce a pressor response. When repeated doses are given tachyphylaxis develops to the pressor action and a depressor response preceding the pressor becomes evident with the second dose and thereafter increases in intensity with each subsequent dose. This author (1, 2) and others (3) have explained this depressor response as being due mainly to myocardial depression.

This paper reports the results of further studies on this depressor action of these aliphatic amines. While most of the experiments were conducted on Oetin a few studies on EA-1 and Tuamine indicate that these compounds act in a similar manner.

PROCEDURE. Healthy mongrel dogs of both sexes were used. Some of the animals were etherized and the others were narcotized with 5 mgm. per kgm. of morphine sulfate. Blood pressure was recorded from either the carotid or femoral artery by means of a mercury manometer or with a "Hypodermic" manometer (4). Peripheral blood flow was measured in the hind leg of some of the animals by means of a Soskin (5) type "bubble" flowmeter inserted into the femoral artery. Such animals were heparinized. Atropine sulfate in a sufficient quantity to prevent the hypotensive effect of a small dose of acetylcholine was administered to each animal. The amines were administered intravenously in the form of their water soluble salts. All doses are expressed as mgm. per kgm.

RESULTS. It has already been shown (1) that tachyphylaxis develops readily to the pressor action of Oetin when doses of 1 or 2 mgm. are used. If the dose is reduced to 0.1 or 0.2 mgm. tachyphylaxis does not usually occur and the depressor response is not present (fig. 1).

Ephedrine has been found to more or less completely block the pressor action of Oetin. Fig. 1 shows the influence of 0.1 mgm. of ephedrine on the response to a small dose of Oetin. The pressor response is removed and no depressor response occurs as this does not usually occur with this dose of Oetin. If a larger dose of Oetin is given after this dose of ephedrine the typical depressor response occurs (fig. 2).

When 1 to 5 mgm. of Oetin are given following 1 to 10 mgm. of ephedrine the ephedrine removes only the pressor response and has little if any influence on the depressor action of Oetin (fig. 3).

was therefore roughly predictable from a) the proportions of ion and free acid, determined by the pK of the substituent group, and b) the varying molar activity of the ion. As anticipated, strongly acidic compounds, yielding no significant amounts of free acid even at pH 5.0, failed to show the usual pH effect.

4. The relative inactivity of the salts of these compounds reflected the fact that they were bound by the trypanosomes to only a minor degree, while the undissociated free acids, like all highly active compounds, were concentrated as much as three hundred-fold. Variations in pH, affecting the relative proportions of ions and free acid, similarly affected the degree to which a given arsenical was bound by the trypanosomes.

5. The question as to whether the undissociated free acids are more strongly bound because of a greater reactivity with cellular constituents, or whether they differ from the ionized salts primarily in their ability to penetrate into the interior of the cell, is discussed in the text. The weight of evidence is in favor of the latter view.

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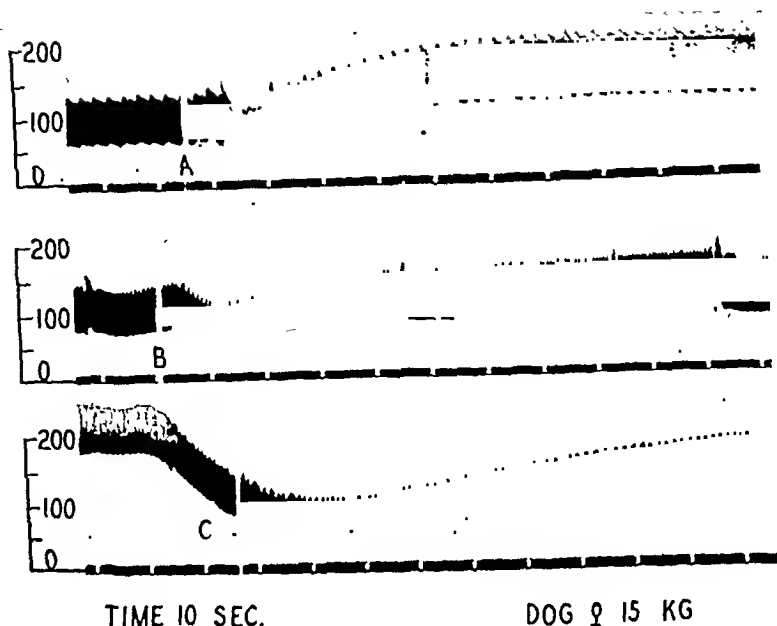
FIG. 3. BLOOD PRESSURE RECORDED WITH "HYPODERMIC" MANOMETER
A—Octin 1 mgm., B—Octin 1 mgm. given 45 min. after ephedrine 1 mgm., C—Octin 5 mgm. given 10 min. after ephedrine 10 mgm.
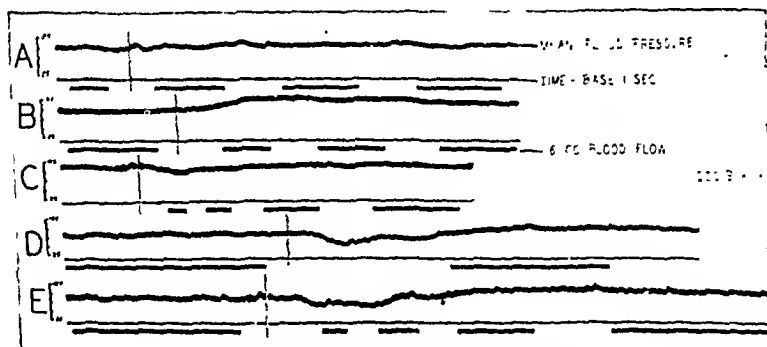
FIG. 4. BLOOD FLOW IN HIND LEG OF A DOG

The mean blood pressure is recorded by means of a "Hypodermic" manometer with the needle placed in the outflow tube of the flowmeter. Blood flow is indicated by the time necessary for the air bubble to traverse the 6 cc. volume in the flowmeter. This flow time is indicated by the heavy lines under the base line. The longer these lines the slower the bloodflow. The spaces between the flow lines indicate the time necessary for the bubble to traverse the distance between the point of injection and the first mark on the flowmeter. This distance is ... ut a 4 cc. volume. Drug injections at vertical lines.

A—Octin   B—Ephedrine 0.01 mgm. intravenously, C—Octin
0.01 mgm.   is a direct continuation of B), D—Octin 1 mgm.
intravenou.   arterially.

Flowmeter studies of the circulation in the dog's leg show that the depressor response of the aliphatic amines is due in some measure to peripheral vasodilation The first dose of the amine produces a slight vasoconstriction but the second dose or any dose following ephedrine produces only vasodilation. Fig. 4 gives the results of a flow study using ephedrine to block the pressor action of Octin.
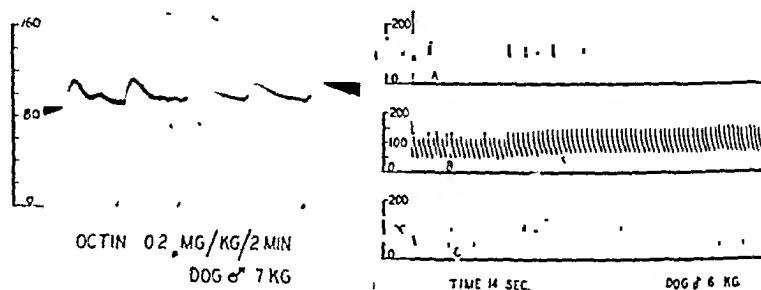
FIG. 1 LEFT—BLOOD PRESSURE RECORDED WITH A MERCURY MANOMETER.
RIGHT—BLOOD PRESSURE RECORDED WITH "HYPODERMIC" MANOMETER
A—Octin 0 1 mgm , B—Ephedrine 0 1 mgm , C—Octin 0 1 mgm
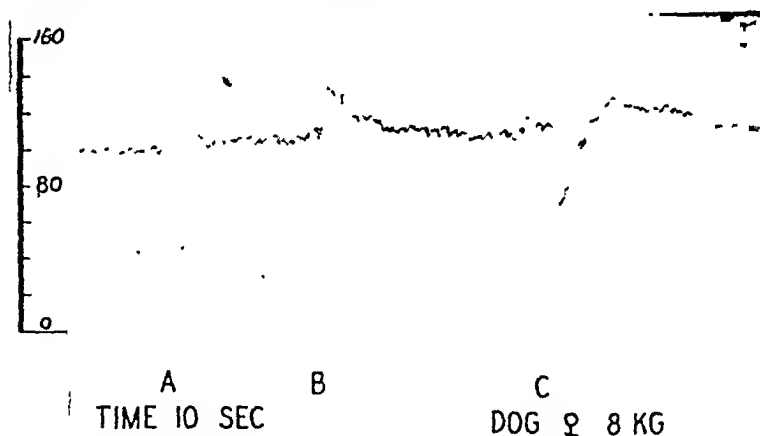
FIG. 2. BLOOD PRESSURE RECORDED WITH A MERCURY MANOMETER
A—Octin 0 1 mgm., B—Ephedrine 0 1 mgm , C—Octin 1 mgm

Peripheral administration (intra-arterial) increases blood flow in the leg while central administration (intravenous) decreases flow during the depressor phase of the drug

Some dogs respond to intravenous epinephrine with a diphasic response. The blood pressure first rises then falls below the control level. Woodbury and Marsh (6) have shown this fall in blood pressure to be due to peripheral vasodila-

fall in blood pressure becomes an important consideration when they are used clinically for their pressor effect. Tuamine has been proposed by Kohlst edt and Page (8) as a pressor compound to be used in conjunction with intra-arterial blood transfusion in the treatment of hemorrhagic shock. EA-1 has been used by Roman-Vega and Adriani (9) as a pressor agent to prevent the hypotension associated with spinal anesthesia. If these amines are given repeatedly or are given after ephedrine there is a good possibility that a fall in blood pressure will result. Overstimulation of the heart or direct myocardial depression could also produce very undesirable reactions.

SUMMARY

1. The three aliphatic amines studied produce a depressor response in dogs which is due in part to peripheral vasodilation.

2. This vasodilation is produced in a manner similar to that of the aromatic sympathomimetic amines such as epinephrine.

3. The pressor action of these aliphatic amines is more or less completely blocked by ephedrine.

4. If these aliphatic amines are to be used clinically for their pressor effect not more than one dose should be used and they should probably not be used with ephedrine.

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tion. A typical diphasic epinephrine response is shown in fig. 5. If Octin is administered to this type of animal the depressor response to epinephrine is greatly enhanced. In dogs that show only a pressor response to epinephrine Octin then potentiates the rise in blood pressure. The second dog in fig. 5 does not show the typical diphasic epinephrine response. The pressor effect, however, is very fleeting and in this animal Octin potentiates both the pressor and depressor effect of the epinephrine.

DISCUSSION. The first dose of any of these aliphatic amines is mainly pressor in action; the increase in blood pressure being due chiefly to cardiac stimulation and to a lesser extent to peripheral vasoconstriction. This cardiac stimulation is apparent in pulse contours recorded following Octin administration. It has also been demonstrated with EA-1 by Jackson (3) and by Heringman and Adriani (7). Kohlstaedt and Page (8) have shown the cardiac stimulating action of Tuamine. Blood flow studies in this paper have indicated the slight vasoconstricting effect of these amines in the dog's leg.
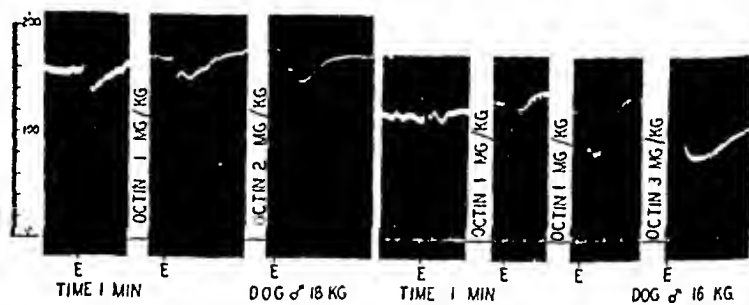
FIG. 5. BLOOD PRESSURE RECORDED WITH A MERCURY MANOMETER
E—Epinephrine 0 005 mgm

When the pressor action of these amines is blocked by repeated doses of the amine or by previous administration of ephedrine the depressor action becomes apparent. Flow studies show that peripheral vasodilation is present during the depressor phase. Some of the depressor action can also be due to direct myocardial depression as fatal doses of these amines in anesthetized animals usually produces death by cardiac failure.

The mechanism of the vasodilating action of these aliphatic amines is probably similar to that of the aromatic sympathomimetic amines such as epinephrine. The vasodilation is not influenced by atropine. Ergotoxine has been found to have no influence on either the pressor or depressor actions of these amines. Jackson (3) found evidence that EA-1 potentiated the depressor response of epinephrine following ergotoxine. In animals that show a marked vasodilation with epinephrine these aliphatic amines will potentiate the epinephrine depressor effect.

The ability of these aliphatic amines to produce peripheral vasodilation and a

THE EXCRETION OF SODIUM TRICHLORACETATE

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It has been shown that trichloracetic acid is present in the urine of dogs (Barret, Cunningham & Johnston, 1939) and of human subjects (Powell, 1945) after trichlorethylene anaesthesia. In the latter investigation, analyses of blood and urine suggested that part of the trichlorethylene absorbed is fixed and metabolized in the tissues. This is followed by diffusion of the metabolite, trichloracetic acid, into the bloodstream where it reaches a maximum concentration in 48 hours, and is then slowly excreted in the urine over a period of 15–20 days.

Barret, Cunningham & Johnston (1939) have observed that trichloracetates administered subcutaneously to rabbits are excreted very slowly, being present in small amounts in the urine two weeks after the injection.

It seems, therefore, that the elimination of trichloracetates is a long process, whether these substances are produced in the tissues or are introduced from outside into the circulation. To obtain quantitative data on this point, we have determined the rate of excretion of sodium trichloracetate, by human subjects, after intravenous administration of the salt.

METHODS. Sodium trichloracetate in amounts varying between 1.5 and 3 g. was given in 3 per cent aqueous solution, which was found to be approximately isotonic. It was administered to six patients by intravenous drip over periods of approximately 1 hour.

Blood samples were collected at intervals and analysed by a method based on the Fujiwara reaction. This method involves protein precipitation, followed by treatment of the filtrate with pyridine and alkali. Under these conditions, trichloracetates give a purplish red colour, which is measurable down to a concentration of 1 mg./100 ml. blood (Powell, 1945).

Urine was collected 24-hourly and analysed by the same method.

RESULTS. Blood concentrations of trichloracetate observed 5 minutes after the injection varied between 10 and 22 mg./100 ml., depending on the size of the patient, and the amount of trichloracetate injected. No ill effects were observed, but during the first few hours after the injection the patient became very drowsy—an effect of trichloracetates observed by Liebreich (1869).

During the first 30 hours after the injection, the blood concentration had fallen to about 50 per cent of the initial value. This was partly accounted for by diffusion into the tissues, since only about 20 per cent of the amount injected had appeared in the urine. The blood concentration then decreased very slowly, having fallen to 30 per cent of the initial value in 90 hours and to 20 per cent in 140 hours. Blood specimens obtained 10 days after the injection still contained 2 to 3 mg. of trichloracetate/100 ml., which was carried entirely in the plasma.

The amount of trichloracetate in the urine decreased correspondingly. At the end of the tenth day approximately 75 per cent of the total amount injected had appeared in the urine. The data obtained from two patients who received 1.8 and 3 g. of sodium trichloracetate respectively are summarized in figures 1 and 2.

TABLE 1

Distribution of trichloracetate in plasma and extracellular fluid

TIME	TRICHLORACETATE CONCENTRATION		TOTAL TRICHLOR-ACETATE	CALCULATED EXTRACELLULAR FLUID
	Blood	Plasma-water		
Patient 1. Weight 45.6 Kg. Haematocrit 39%. 1.7 g. sodium trichloracetate injected				
hours	*mg /100 ml*	*mg./100 ml.*	*g*	*litres*
10	8.0	14.0	1.51	10.8
34	5.7	10 0	1.24	12.4
58	4.4	7.7	1.00	13.0
82	3.5	6.1	0.77	12.6
106	2.8	5.0	0.64	12.8
130	2.4	4.2	0.54	12.9
154	2.1	3.7	0.46	12.4
Mean. = 27% body weight		.		12.4
Patient 2. Weight 48 2 Kg. Haematocrit 40% 2 9 g. sodium trichloracetate injected				
34	10.2	17.9	2 00	11.2
58	7.8	13.6	1.64	12.0
82	6.2	10.8	1.30	12.0
106	4.8	8.4	1.10	13.0
130	4.0	7.0	0.98	14.0
Mean = 26% body weight		. .		12.4
Patient 3. Weight 64 Kg. Haematocrit 45%. 1.8 g. sodium trichloracetate injected				
34	6.5	11.9	1.36	11.4
58	5.0	9.5	1.02	10.8
82	4.4	8.3	0.91	11.0
106	3.2	6.1	0.80	13.1
Mean = 18% body weight		.		11.6

DISCUSSION. The trichloracetate ion does not penetrate into the red cell, and is therefore probably distributed throughout the body in the extracellular fluid. Assuming that the distribution is uniform, it should be possible to calculate approximate values for the extracellular fluid volume from the amount of trichloracetate remaining in the body and the concentration of trichloracetate
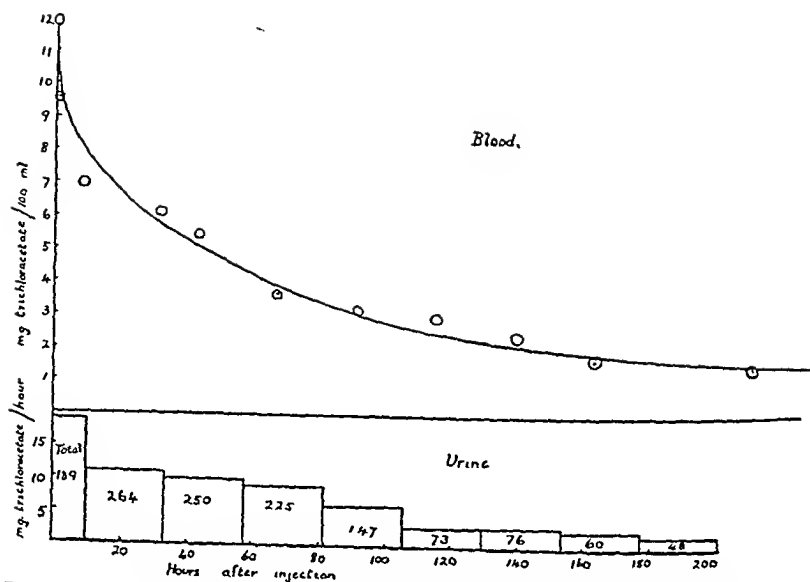
FIG. 1. BLOOD CONCENTRATIONS AND URINARY EXCRETION OF SODIUM TRICHLORACETATE AFTER AN INTRAVENOUS INJECTION OF 17 g.
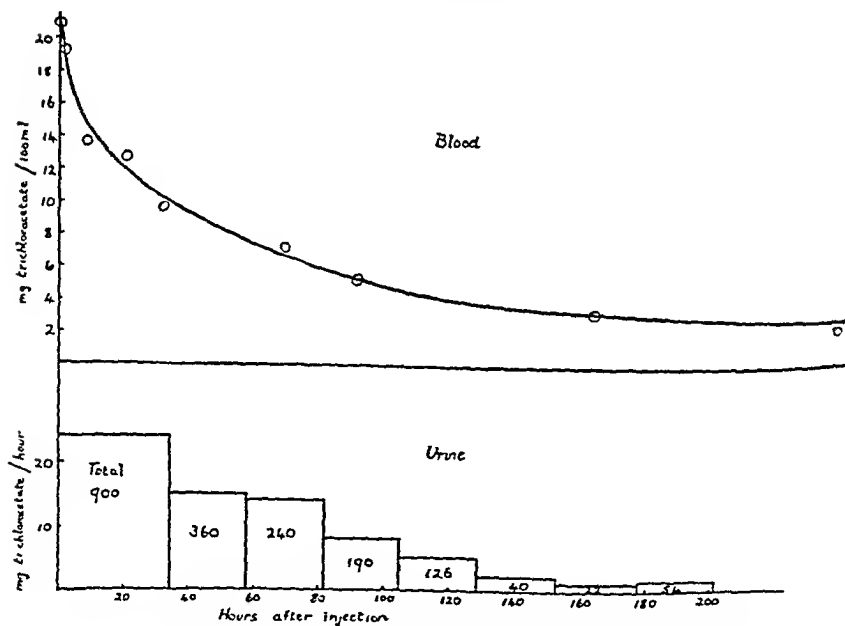
FIG. 2. BLOOD CONCENTRATIONS AND URINARY EXCRETION OF SODIUM TRICHLORACETATE AFTER AN INTRAVENOUS INJECTION OF 29 g.

In figure 3, the curve drawn is that given by

$$c = \frac{2}{3} e^{-0.0085t} + \frac{1}{3} e^{-0.13t}$$

which was derived by trial and error. This expression fits the experimental results reasonably well.

SUMMARY

The excretion of sodium trichloracetate after intravenous injection is slow. Approximately 75 per cent of the amount injected has appeared in the urine by the tenth day after the injection. An expression can be derived theoretically which will fit the time/blood concentration curves.

There are indications that the trichloracetate ion is distributed throughout the body in the extracellular fluid.

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in the plasma-water. Taking the results given in fig. 1, and calculating the extracellular fluid volume at 10 hours and then at 24-hourly intervals, we obtain values between 10 8 and 13.0 litres, with a mean of 12.4 litres. The results in figure 2 give values between 11.2 and 14.0 litres, with a mean of 12.4 litres (table 1). These values represent 27 per cent and 26 per cent respectively of the total body weight. The results obtained from a third patient are also given in table 1—here the calculated extracellular fluid volume is rather low, 18 per cent of the body weight. These values agree fairly well with those obtained after injection of bromides by Winkler and Smith (1938) and Brodie, Brand and Leshin (1939). In the other three patients, unfortunately the amounts of trichloracetate given were not accurately measured, so that here it was not possible to calculate values for the extracellular fluid volume.
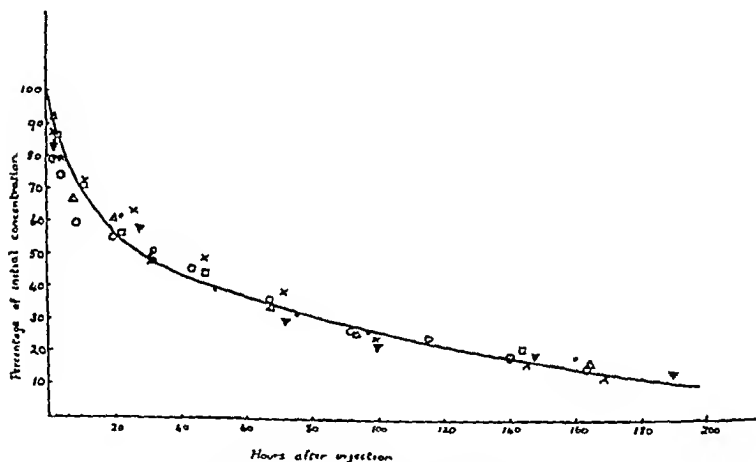
FIG. 3. BLOOD CONCENTRATIONS OF SODIUM TRICHLORACETATE IN SIX PATIENTS AFTER INTRAVENOUS INJECTION OF 1 5 TO 3 g

The shape of the excretion curve is essentially the same for each patient, as is shown in figure 3, where blood concentrations are expressed as percentages of the concentration found 5 minutes after the injection. The experimental points show considerable scatter during the first few hours after the injection. It is probable that a more uniform set of data would have been obtained with a shorter, or at least a standard, injection period.

The experimental results cannot be represented by a simple exponential expression, even after the initial steep decline. By assuming a first order rate of excretion and of transfer between blood and tissues, an expression of the type

$$c = Hc^{at} + Je^{\beta t}$$

can be derived theoretically (Teorell, 1937), to give the decrease of blood concentration c, with time t. Here, H, J, α and β are factors which contain the velocity-constants of the diffusion processes involved.

The preparation used both orally and parenterally was 2-méthyl-1,4 naphtho-hydroquinone diphosphoric ester tetra sodium salt (Synkayvite). For oral administration 25 mg. tablets and for parenteral medication 2 cc. ampuls containing 38 mg./cc. were employed. Both forms were specially prepared.

Oral treatment was given to 14 patients of whom 13 were hypertensive and one served as control. Each subject received 25 mg. of the hydroquinone a day for periods varying from 3–6 weeks (average 4 weeks). The total amounts administered per patient ranged from 525 mg. to 1050 mg.

The parenteral form of the vitamin K-like compound was given intravenously to nine patients of whom seven were hypertensive and two had normal blood pressure. Four hypertensive subjects and the two normal controls received individual injections, 38 mg. each, either daily or on alternate days, for four to eight doses. Three hypertensive cases were given massive single doses, 152 mg. each. The total amounts administered parenterally per patient ranged from 152 mg. to 304 mg.

The 19 subjects had 23 separate courses of the drug with four hypertensive individuals being changed from oral medication to parenteral therapy. The results were as follows:

ORAL GROUP

13 Hypertensive cases
  Four (E) responses (2 males, 2 females)
  Two (F) responses (1 male, 1 female)
  Seven (N) responses (3 males, 4 females)
1 Normal control
  One (N) response (male)

PARENTERAL GROUP

A. Individual intravenous injections
  4 Hypertensive cases
    Two (E) responses (2 females)
    One (F) response (male)
    One equivocal response (male)
  2 Normal controls
    Two (N) responses (1 male, 1 female)
B. Single massive intravenous injection
  3 Hypertensive cases
    One (E) response (male)
    Two (N) responses (1 male, 1 female)

DISCUSSION OF RESULTS. In the group of thirteen hypertensive patients receiving Synkayvite by mouth there were four (E), two (F), and seven (N) responses. The blood pressure of the normal control was not affected by oral medication.

In four hypertensive subjects receiving Synkayvite intravenously there were two (E), one (F), and one equivocal response. In the latter patient there had

THE BY-EFFECTS OF ANTI-HEMORRHAGIC QUINONES

II. Antipressor Action in Chronic Hypertension in Man

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In the course of our investigations of the effects, other than those upon the prothrombin-producing mechanism, of antihemorrhagic quinones, it was noted in several subjects with chronic hypertension, that the blood pressure became reduced after administration of large doses of vitamin K-like quinone. It was decided consequently to determine whether these drugs possessed antipressor activity.

MATERIAL, PROCEDURE AND OBSERVATIONS. The clinical material at the Goldwater Memorial Hospital is especially suited for such work because all of the cases are of the chronic type, and have been under supervision for many months during which periods their respective blood pressure curves had been well established.

Nineteen patients constitute the present series. Seventeen of these had hypertension of at least several years' standing. Two of the latter had associated heart block with Adams-Stokes syncope. In fifteen the diagnosis was essential hypertension. The remaining two patients were normal from the standpoint of the cardiovascular-renal systems and their history did not reveal any past significant elevation of the blood pressure. These two patients served as controls.

Measurements of the blood pressure were made almost daily by the same observer (N. R.). The terminal change of sound (fourth phase) was used as the point of diastolic reading. Averages for a control period were made after a minimum of eight, and usually more days of observation. Despite the frequently emphasized statement that large variations in pressure fluctuations are common in hypertensive patients from day to day, it was found that the mean pressures over two control periods in the same person approximated each other to a striking degree, especially the diastolic component. It is important to note that all of the patients used in the present study were either bed-ridden or chair-ridden. A few were allowed to move about the ward and to visit the lavatory, but the overall picture was that of a group of individuals with remarkably constant day to day habits and limited activities. The criteria of significant deviations were made sufficiently rigid to exclude the possibility of merely fortuitous variations being responsible for positive findings.

Arbitrarily the following criteria were decided upon for the evaluation of the results. A fall of thirty millimeters of mercury in the average systolic readings or one of eighteen millimeters for the diastolic pressure was classified as an excellent (E) response. A reduction of twenty millimeters in the average systolic or ten millimeters in the average diastolic pressure was classified as a fair (F) response. Less than these values were considered as a non-response (N).

currence of the acute venous thrombosis nor did any other thrombosis become evident. Thus, it is our experience that administration of antihemorrhagic quinone at high dosage levels is without danger of thrombosis even in subjects within the sclerotic age, and who have recently had thromboses.

Symptoms of hepatic demage such as icterus or biliuria did not appear in any of the cases. Renal function studies including the blood urea nitrogen, urinary concentrating capacity and search for the appearance of abnormal constituents in the urine yielded no deviations from the premedication status. The cytology and hemoglobin content of the blood remained unchanged. The ventricular rate was unaffected. The hematocrit values showed no significant alterations.

The mechanism of the antipressor action of the drug is at present within the realm of conjecture. It is not a rapidly acting vasodilating agent. This is adequately demonstrated by the fact that there was no reduction of blood pressure when measured every few minutes or even hours after a large intravenous dose. The possibility that a degradation product of the naphthohydroquinone, or possibly a conjugation product or some other compound liberated by the particular metabolism of this substance within the organism, might be responsible for the depressor effect is supported by the latent period which invariably elapses before antipressor activity becomes manifest. Attention is drawn at this point to a theoretical consideration of similar effects exerted by oxidation products of pressor amines recently presented by Oster (2) and applied by others (3) (4). We are attempting to establish whether or not products possessing antipressor activity are excreted in the urine after the administration of vitamin K-like compounds (5).

The antipressor activity of the drug seems to be independent of its prothrombin-promoting action. Prothrombin times estimated in both the whole and the diluted (12.5%) plasma were carefully studied in all of the patients and no parallelism between these and the blood pressure changes could be detected. Dicumarol in adequate doses when given before or after the synthetic vitamin K radically altered the prothrombin-clotting times but presented no evidence of causing any aberration of the blood pressure responses.

Little can be said at this time of the possible alleviation of any of the common symptoms or sequelae of vascular hypertension. Three of the patients who complained of moderate headaches were relieved after reduction of their respective blood pressure levels. One other patient who revealed a favorable response suffered accentuation of her headaches following intravenous injection of large doses of the drug.

It is still undecided whether the elevated blood pressure is cause or effect in this disease process. Consequently, whether prolonged antipressor effect capable of continued reduction such as herein described is symptomatic or fundamental therapy is an important question awaiting further elucidation.

Although the present series is not large, nevertheless, the favorable responses observed in some cases of chronic and fixed hypertension appeared striking enough to warrant this report.

occurred a significant reduction in blood pressure after Synkayvite orally and the lower tension was maintained when parenteral therapy replaced the oral medication. Later, however, the blood pressure rose to a higher level after the drug was withdrawn. Two cases with normal arterial tension showed no alteration in blood pressure after intravenous injection of the antihemorrhagic compound. Three patients given single massive doses intravenously revealed the following responses: one (E) response in the systolic phase only and two (N) responses. Three hypertensive cases which disclosed (N) responses after oral administration showed (E) responses when they were later given the drug intravenously.

The best results in reducing blood pressure seemingly attended intravenous administration. The doses given were arbitrary ones; the optimal therapeutic dose and the optimal maintenance dose remain to be determined. Although a dose of 38 mg. per day parenterally was found to be effective in most cases, we believe lesser quantities will prove adequate in the majority of instances. It appears that the susceptibility of the patient is of greater importance in determining his response than the magnitude of the dose administered since both excellent and negative results were observed with the smallest as well as with the largest quantities used. Normal blood pressure was not altered by the drug

Reduction in vascular tension, when it occurred, became evident only after a latent period of at least one but generally more days. If in patients who had responded favorably the administration of the drug was halted, the pressure usually rose but rarely to the initial level. In those instances in which the drug was without effect discontinuance of therapy was followed by no alteration in the established hypertension. Occasionally there was noted an indication of a release phenomenon: after having descended, the pressure rose despite the continuation of the drug, but the level of the mean pressure which prevailed during the premedication control period, was rarely reached. Another interesting change was a greater mobility of the day to day blood pressure readings after the therapy was commenced as compared with the daily values obtained during the control period. These latter readings were more constant as demonstrated by an almost horizontal line generally obtained when the premedication findings were plotted.

As a consequence of the therapy the prothrombin time was reduced in a number of instances to hyperprothrombinemic levels. Despite this, none of the cases yielded evidences of thrombotic phenomena. Identical experiences in animals and man were described in an earlier report of this series (1). The implication is that in addition to mere augmentation of the coagulability of the blood other factors are essential to induce intravascular thrombosis. An extensive clinical experience supports this thesis and the following observations serve to illustrate it. A forty-year old male presented an acute venous thrombosis involving the left saphenous tract. Marked reactive hyperprothrombinemia was demonstrated. Dicumarol therapy was followed by rapid subsidence of the acute process and eventually by restoration of the prothrombin time to normal. An intravenous injection of forty milligrams menadione bisulfite was given a fortnight later. The prothrombin time returned to and remained at the initial hyperprothrombinemic level for three days However, there was neither re-

THE ABSORPTION OF CINCHONA ALKALOIDS IN THE CHICK AND ITS RELATIONSHIP TO ANTIMALARIAL ACTIVITY

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In view of the possibility of synthetic cinchona alkaloids becoming available, it seemed that a survey of the metabolism and relative antimalarial activities of the alkaloids and their modifications would be helpful in planning future chemical research. The work described here was designed to investigate the problem of how far the absorption, red blood cell and plasma concentrations and rates of destruction of a group of cinchona alkaloids and derivatives could be correlated with their antimalarial activity in chicks infected with *Plasmodium gallinaceum*.

Buttle, Henry and Trevan (1) and Buttle, Henry, Solomon, Trevan and Gibbs (2) have compared the antimalarial activities of a large number of cinchona derivatives against malaria infections in canaries. Seeler, Dusenberg and Malanga (3) compared the activities of the four natural alkaloids against infections of *P. lophurae* in Pekin ducklings.

Determinations of the distribution of quinine in the tissues of the adult fowl have recently been made by Kelsey, Oldham and Geiling (4). Fulton (5) and Hegner, Shaw and Manwell (6) determined the partition of quinine between red cells and fluid medium by suspending washed red cells in physiological saline containing the alkaloid.

Ramsden, Lipkin and Whitley (7) first recognized the liver as the chief centre of metabolic destruction of quinine. Lipkin (8) showed the reaction to be an oxidation process, and extracted from liver tissue an enzyme which promoted the reaction. The distribution of "quinine oxidase" in the tissues of a number of animals, including the domestic fowl, has been investigated by Kelsey and Oldham (9).

MATERIALS. 1. *Cinchona alkaloids.* The following alkaloids and derivatives were used in the investigations: I. Quinine (*l*) and quinidine (*d*); II. cinchonidine (*l*) and cinchonine (*d*); III. modified alkaloids, niquine (*l*) and niquidine (*d*); IV. saturated products, dihydroquinine and dihydroniquidine.

$$CH_3O \cdot Q \cdot CHOH \cdot HC{\rule{1cm}{0.4pt}}N{\rule{1cm}{0.4pt}}CH_2$$

I
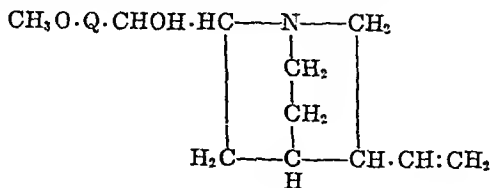
CONCLUSIONS

A significant reduction in blood pressure occurred in about fifty percent of fifteen cases of chronic hypertension of the essential type following the oral or intravenous administration of 2-methyl-1,4 naphthohydroquinone-diphosphoric ester tetra sodium salt (Synkayvite).

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The mixture was incubated for 2 hours at 40°C., with occasional stirring. Enzyme activity was then arrested by adding 20 ml. of 4 per cent NaOH and heating on a steam bath, followed by extraction of the unchanged alkaloid.

(e) *Extraction of alkaloids from biological material.* Blood or plasma samples were diluted with 50 ml. water. 20 ml. 2 per cent NaOH was added and the mixture heated on a steam bath for 30 min. Tissue samples (gut) were heated in 2 per cent NaOH (50 ml. per 5 g. of tissue) until completely disintegrated. Tissue suspensions (liver) were heated until a clear solution was obtained. The alkaloid was then extracted by the modified method of Kelsey and Geiling (14) described by Marshall and Rogers (15). The final acid extraction was made with HCl or H_2SO_4, according to the method of determination of the alkaloid.

Difficulty was experienced in the extraction of niquine. It was found that if niquine was heated with 2 per cent NaOH in the presence of biological material, either fresh or previously heated with the soda, 50–80 per cent of the alkaloid was lost. If, however, the niquine was added to cold NaOH in the presence of fresh or previously heated biological material, and extracted without further heating, all the alkaloid was recoverable. A "cold extraction" procedure was therefore evolved for all determinations of this alkaloid. Blood or plasma samples were diluted with 50 ml. water in a separating funnel. 20 ml. 2 per cent NaOH and 2 ml. acetone were added, and the mixture extracted with 50 ml. ether without previous heating. A troublesome gel formed when whole blood was used, but this was removed by swirling the funnel to aid separation of the phases. The lower viscous layer was run off a little at a time, taking care that no bubbles of ether were entrapped. Gut was ground in a mortar with broken glass, suspended in water sufficient to produce 245 ml. and 5 ml. 0.1 N H_2SO_4 was added to keep the alkaloid in solution. 10 ml. of this suspension was extracted as above. For liver suspensions, an equal volume of 4 per cent NaOH was added and the mixture extracted as for blood.

(f) *Determination of alkaloids.* Three methods of determination were employed, depending on the properties of the particular alkaloid and the amount expected to be present in the extract.

(i) *Fluorimetric method.* Used for determination of fluorescent alkaloids in blood and plasma. The washed ether extract was extracted with sufficient 0.1 N H_2SO_4 to produce a final concentration of about 10 μg. of alkaloid in 5 ml. Since a photoelectric fluorimeter was not available, a comparator for visual determination was designed, using an ordinary mercury vapour lamp. The alkaloidal solution was placed in a tube before a U.V. glass window, and a standard solution of the alkaloid added drop by drop to a second tube containing an equivalent volume of 0.1 N H_2SO_4, until the two fluorescences nearly matched. A volume of H_2SO_4 equivalent to the volume of standard was then added to the first tube before running in the last few drops to produce an exact match. Using a standard solution containing 1 μg. alkaloid per ml., the difference in fluorescence produced by 1 drop (i.e. about 0.05 μg. alkaloid) could be detected.

(ii) *Nephelometric method.* Used for determinations of all alkaloids in gut and liver suspensions, in which comparatively large amounts were present. The ether extract was extracted with 10 ml. 0.03 N HCl, and the determination carried out according to the method of Kyker, Webb and Andrews (16). This method was not sensitive to less than 10 μg. of alkaloid.

(iii) *Colorimetric method.* Used for determination of nonfluorescent alkaloids (cinchonine and cinchonidine) in blood and plasma. This method (Marshall and Rogers (15)), designed to meet the demands of the present investigation, was a modification of Prudhomme's (17) eosin method, using bromothymol blue instead of eosin. The method was sensitive to 0.25 μg. of alkaloid.

(g) *Accuracy of methods employed.* In order to see whether the three methods of determination described above gave comparable results, a series of known concentrations of quinine was prepared, and the alkaloid content determined by each method. The results (table 1) show that the methods compare favourably with each other within their limits of sensitivity.

$$Q \cdot CHOH \cdot HC \text{---} N \text{---} CH_2$$

$$CH_2$$

$$CH_2$$

$$H_2C \text{---} C \text{---} CH \cdot CH : CH_2$$

$$H$$

II

$$CH_3O \cdot Q \cdot CHOH \cdot HC \text{---} NH$$

$$CH_2$$

$$CH_2$$

$$H_2C \text{---} CH \cdot CH : CH \cdot CH_3$$

III
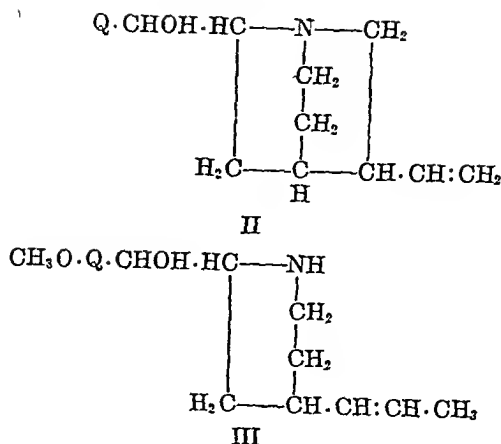
The preparation and structural formula for niquine and niquidine (III) have been described by Henry and Gibbs (10, 11) and Solomon (12). Samples of all the alkaloids used were kindly supplied by Dr. T. A. Henry.

2. *Animals.* The chicks used were of a constant strain, Rhode Island Red x Light Sussex. They were received when one day old and used for antimalarial or absorption tests when 7–21 days old (50–100 g. weight).

3. *Plasmodium strain.* For the antimalarial tests, chicks were infected with a strain of *Plasmodium gallinaceum,* kindly supplied by Dr. F. Hawking of the National Institute for Medical Research, Hampstead.

METHODS. 1. *Metabolism experiments.* (a) *Administration of drugs.* A single oral dose of 50 mgm. of alkaloid base per kgm. body weight was given. Alkaloidal salts were dissolved in distilled water; bases in a minimum amount of N HCl and made up to final volume with water. The solutions contained 0.25 per cent w/v of base. Doses were adjusted according to body weight, and were introduced into the crop from a syringe fitted with a large bore needle with the point smoothed down. One bird was used for the determination of each point on the concentration-time curves.

(b) *Absorption of alkaloids from the gut.* Chicks were killed by ether vapour at varying times after dosing. The entire gut, from oesophagus to cloaca, was removed and dropped into 2 per cent NaOH for determination of unabsorbed alkaloid.

(c) *Concentration of alkaloids in the blood.* Chicks were given doses of alkaloid, killed by ether vapour at varying intervals, and samples of heart blood were removed. Potassium oxalate was used to prevent clotting. 0 5 to 1 ml. of whole blood was taken for determination of alkaloid content, and a small sample for determination of haematocrit value. The remainder was centrifuged and 0 5 to 1 ml samples of plasma were taken for alkaloid determination. The concentration of alkaloids in the red cells was calculated by difference between whole blood and plasma values, and expressed as mgm per 100 ml of red cells, according to the haematocrit value of the sample.

(d) *Destruction of alkaloids by the liver.* Normal chicks were killed without anaesthetic by a blow on the head. The liver was removed, weighed, and ground to a smooth cream in a mortar. The ground tissue was suspended in Krebs and Henseleit's (13) physiological saline solution, strained through muslin, and adjusted to contain 0.5 g. of tissue per 10 ml. To each 10 ml. was added 100 μg of alkaloid, in 1 ml of solution, and 9 ml. of saline solution.

was "destroyed" by chick liver suspensions to a greater extent than the other alkaloids. Cinchonine and cinchonidine were not destroyed at all.

2. *Antimalaria experiments.* In order to allow for variance in the magnitude of the peak infections of control birds, the percentage of red cells parasitized in each treated bird was deducted from the mean percentage cells parasitized in the control group. The difference was called the percentage reduction of infection. The results obtained are shown in table 2. Dose-effect curves were constructed by plotting mean percentage reduction of infection against log. dose (figs. 2A and 2B), and the dose required to produced a 50 per cent reduction of infection was determined. This value was used in expressing the "quinine equivalents" of the alkaloids. It must be borne in mind, however, that these equivalents do not fully represent the relative potencies because the dose-response curves are not all parallel.

The quinine equivalents (table 3) show dihydroquinine to be the most efficient alkaloid in suppressing infections of *P. gallinaceum*, with cinchonine and niquidine next in descending order. Niquine was the least effective. All the alkaloids, excepting niquine, were more effective than quinine.

DISCUSSION. Very little work has been done on the metabolism of cinchona alkaloids and their derivatives in chicks, and in most cases investigations have been limited to quinine. In their determinations on the distribution of quinine in the tissues of the fowl, Kelsey, Oldham and Geiling (4) used adult birds and gave doses of quinine greatly in excess of the dose required to suppress infections of chicken malaria. Indeed, the 400 mgm. per kgm. dose used by these workers approached the toxic level (Boyd (18) reports an oral M.L.D. for quinine HCl in canaries of 400 mg. per kgm.) In the present work, a standard dose of 50 mgm. per kgm. of all alkaloids was used in the experiments on absorption and blood concentration. This dose was approximately the minimum dose of quinine required to clear completely the peripheral parasitaemia in *P. gallinaceum* infections. Young chicks of similar age to those used in the antimalaria tests were used for all metabolism experiments, including those on destruction of alkaloids by liver suspensions. Thus, the conditions under which both the metabolism and the antimalarial experiments were carried out were as nearly identical as possible. The importance of using birds of similar age in these comparative experiments is shown by recent investigations in these laboratories (19) which indicate that quinine is "destroyed" more rapidly by suspensions of young chick's liver than by liver suspensions from older birds. This explains the observation of Oldham *et al.* (20) that lower concentrations of quinine were found in the tissues of chicks than in tissues from grown birds. These workers (20, 21) were unable, however, to demonstrate any "quinine oxidase" activity in chick liver.

Previous work on the passage of cinchona alkaloids into the red cells has in most cases (5, 6) been done by suspending the cells in physiological saline containing the alkaloid. This procedure cannot be expected to give a true picture of the distribution of alkaloid between plasma and red cells since it does not take into consideration "vital" processes (as distinct from simple osmosis) which

Experiments on the recovery of alkaloids added to biological material showed that, provided standard curves prepared under similar conditions were used, recoveries were in most cases within ±5 per cent.

2. *Antimalaria experiments.* Blood from a chick heavily infected with *Plasmodium gallinaceum* was diluted with physiological saline containing 0.1 per cent. "Liquoide" (sodium polyanetholsulphonate—Roche) as anticoagulant. The dilution was adjusted to contain 100 million parasitized red cells in 0.2 ml. Groups of chicks were inoculated intravenously each with 0.2 ml. of blood suspension. Doses of alkaloids in solution were given orally immediately after inoculation, and were repeated twice daily for four days. A range of three or four different doses was given for each alkaloid. Antimalarial activity was assessed on the fifth day (on which control birds reached a peak peripheral infection) by comparison of the percentages of red cells parasitized.

RESULTS. 1. *Metabolism experiments.* The results of the determinations of rates of absorption and blood concentrations are shown graphically in fig. 1. The concentrations of alkaloids in the red cells are expressed numerically in

TABLE 1

Range of sensitivity and accuracy of recovery of quinine by three analytical methods

AMOUNT OF QUININE ADDED	AMOUNT OF QUININE FOUND (μG.)		
	Fluorimetric method	Nephelometric method	Colorimetric method
μg.			
0.07	0.068	Nil	0.11
0.15	0.152	Nil	0.31
0.4	0.400	Nil	0.40
0.8	0.79	Nil	0.70
3.6	3.58	Nil	3.50
17	18.0	14.0	17.8
38	40.0	40.0	38.0
105	107	106	101
188	190	184	190

table 3 as the areas enclosed by the red cell concentration-time curves up to the time when the concentration reached zero. (Where the last determined point was not quite zero, the curve was produced to meet the baseline and the total enclosed area determined). Table 3 also shows the relative rates of absorption from the gut and rates of destruction by suspensions of chick liver. All the values for destructions were obtained from the same liver sample.

Cinchonidine and dihydroniquidine were the most slowly absorbed alkaloids, while quinidine was the most rapidly absorbed. The blood concentration curves of the four natural alkaloids were characterized by a sharp peak in the red cell curve one hour after the dose. Niquidine and dihydroniquidine showed the most prolonged blood concentration curves. Comparing the areas enclosed by the red cell concentration-time curves, which takes into consideration the length of time the concentration was maintained, dihydroniquidine showed the highest concentration, followed by quinidine and cinchonine. Niquine appeared in the red cells only during the first half hour after administration. Quinine

QUINIDINE
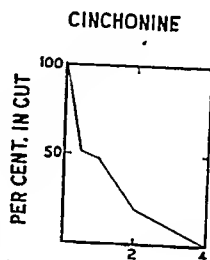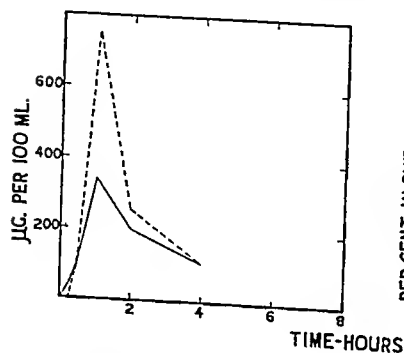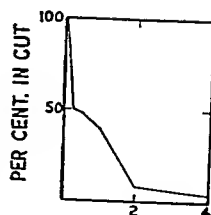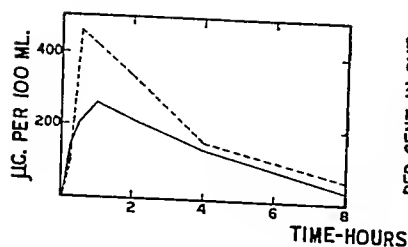
CINCHONINE

NIQUIDINE

DIHYDRONIQUIDINE

a

FIG. 1 (*Continued*)

b
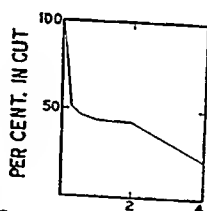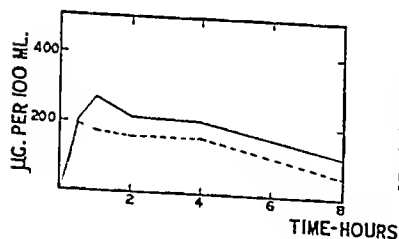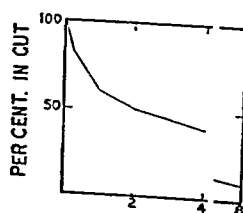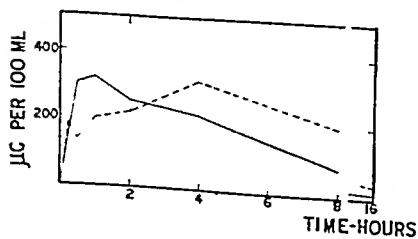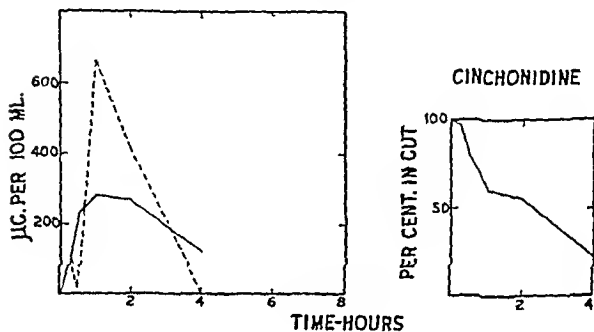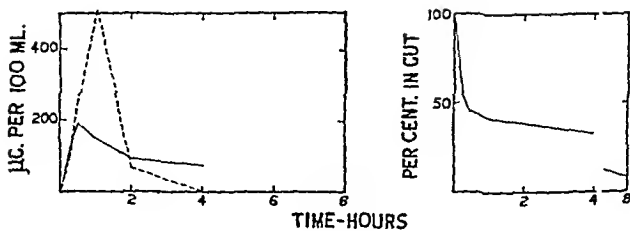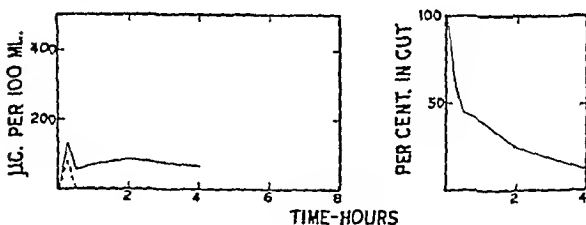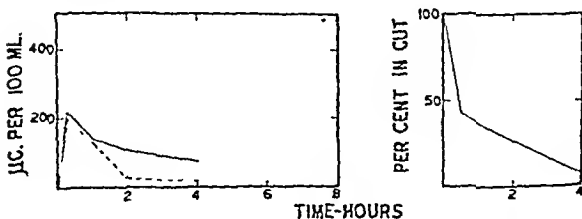
FIG. 1. METABOLISM OF CINCHONA ALKALOIDS IN CHICKS

(a) Blood concentration curves. Continuous line, whole blood, broken line, red cells.
(b) Rate of absorption of alkaloids from the gut.
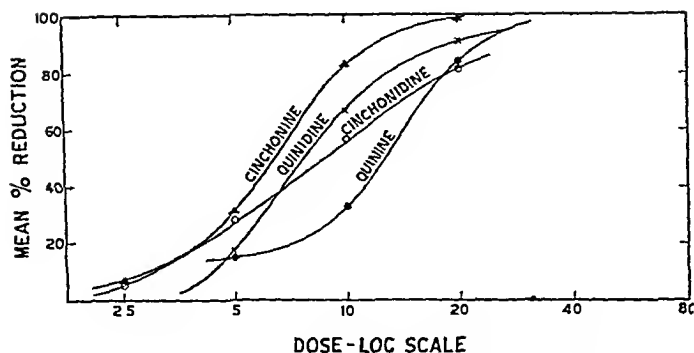
Fig. 2A. Antimalarial Action of Natural Alkaloids
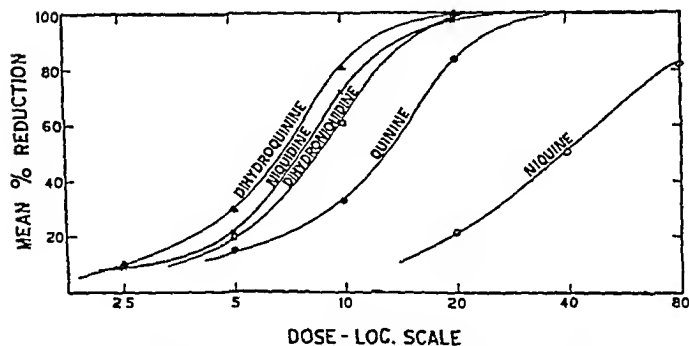
Fig. 2B. Antimalarial Action of Cinchona Derivatives

TABLE 3

Summary of results of metabolism and antimalarial experiments

ALKALOID	ANTIMALARIAL ACTIVITY (QUININE EQUIVALENT)	PER CENT ALKALOID ABSORBED FROM GUT AFTER 2 HOURS	CONC ALKALOID IN RED CELLS (AREA OF CONC-TIME GRAPH)	μG ALKALOID DESTROYED BY 0 5 G. CHICK LIVER IN 2 HOURS
Quinine (*l*).	1.0	62	150	77
Quinidine (*d*)	1.6	92	422	35
Cinchonidine (*l*)	1.5	40	291	2
Cinchonine (*d*) .	1.9	80	327	0
Niquine (*l*)	0.3	75	5	38
Niquidine (*d*)	1.7	57	285	46
Dihydroquinine (*l*)	2.0	73	68	28
Dihydroniquidine (*d*)	1 6	50	723	34

probably influence the distribution of alkaloids *in vivo*. All our determinations of red cell concentrations were therefore made on freshly drawn blood from birds given oral doses of the alkaloids.

TABLE 2

Results of antimalarial experiments

ALKALOID	DOSE (8 DOSES GIVEN)	NO. OF BIRDS	MEAN REDUCTION OF INFECTION	DOSE TO CAUSE 50% REDUCTION	MAXIMUM SLOPE OF CURVE
	mg /kgm.		*per cent*	*mg./kgm.*	
Quinine (*l*)	5	39	14.6		
	10	41	32.8	13.0	190
	20	38	83.6		
Quinidine (*d*)	5	15	17.3		
	10	14	66.6	8.1	190
	20	15	91.0		
Cinchonidine (*l*)	2.5	4	4.8		
	5	21	26.5	8.4	110*
	10	17	57.0		
	20	20	81.4		
Cinchonine (*d*)	2.5	5	6.4		
	5	16	28.5	6.7	210*
	10	16	83.6		
	20	15	99.6		
Niquine (*l*)	20	9	21.6		
	40	13	50.4	40.0	140*
	80	12	92.2		
Niquidine (*d*)	2.5	5	8.8		
	5	12	21.2	7.7	210*
	10	13	71.7		
	20	13	98.1		
Dihydroquinine (*l*)	2 5	5	9.0		
	5	18	29.6	6.6	190
	10	19	81.1		
	20	17	100.0		
Dihydroniquidine (*d*)	5	21	19.8		
	10	23	61.1	8.7	190
	20	17	99 5		

* Slopes differ significantly.

The use in these investigations of three entirely different methods of determination of alkaloids might be open to criticism. The reasons for this were (a) that the small blood samples from young chicks and the small dose of alkaloid given

SUMMARY

1. Determinations of the rate of absorption from the gut, red cell concentrations and antimalarial activity in chicks of the four natural alkaloids of cinchona and four derivatives showed that antimalarial activity was, in most cases, correlated with rate of absorption and red cell concentrations.

2. In three optically active pairs of alkaloids, the d-isomers showed a greater rate of absorption, higher red cell concentrations and higher antimalarial activity than the l-isomers.

3. The rate of "destruction" of the alkaloids by chick liver suspensions was not related to optical configuration. Cinchonine and cinchonidine, unlike the other alkaloids, were not metabolized at all.

4. Saturation of the double bond in quinine doubled the antimalarial activity. Dihydroniquidine, however, showed no greater activity than niquidine.

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made it necessary to use the more sensitive fluorimetric method for blood concentrations; (b) that none of the hitherto published non-fluorimetric methods was sufficiently sensitive to determine the blood concentrations of cinchonine and cinchonidine, for which it was necessary to devise the new colorimetric method; and (c) that the nephelometric method was the most convenient where comparatively large amounts of alkaloid were present. Reference to table 1 shows that the three methods of analysis were equally accurate within the limits in which they were employed. It should be noted that one method of *extraction* of the alkaloids from biological material was used throughout.

Comparison of the results of the metabolism and antimalaria experiments (table 3) shows that, generally speaking, antimalarial activity can be correlated with the rate of absorption and red cell concentrations of the alkaloids. Of the two isomeric pairs of natural cinchona alkaloids, the dextrorotatory isomers were absorbed from the gut more rapidly, produced higher red cell concentrations and were more actively antimalarial than the laevorotatory isomers. The derivatives, niquine and niquidine behaved similarly except that the absorption from the gut was more rapid in the *l*-isomer, niquine. The rate of "destruction" of the alkaloids by the liver appears not to be influenced by optical configuration, the most noticeable feature being that cinchonine and cinchonidine were not metabolized at all by liver suspensions.

The saturated derivatives, dihydroquinine and dihydroniquidine were more irregular. Dihydroquinine was the most effective antimalarial of the whole group, though its concentration in the red cells was relatively low. From the prolonged blood concentration produced by dihydroniquidine, it would have been expected to possess a higher activity.

The results of the antimalarial tests upon chicks were not in agreement with the reports of previous workers, using other species of plasmodium and other hosts. Buttle, Henry *et al.* (1) reported that there was no obvious relationship between optical activity and antimalarial action, though they found that, of the four natural alkaloids, the *l*-isomers were more active. Buttle *et al.* (2) expressed surprise that the *d*-isomer, niquidine, was more active than the *l*-isomer, niquine. Seeler *et al.* (3) reported that there was no appreciable difference in activity between the four natural alkaloids tested against *P. lophurae* infections in ducklings. These discrepancies might be explained by different rates of absorption and distribution of the alkaloids in different hosts

The results of these investigations show that efficiency of the alkaloids of cinchona and some of their derivatives in suppressing infections of *P. gallinaceum* in chicks is governed to a large extent by their respective rates of absorption and blood concentrations. Of isomeric pairs tested, the *d*-isomers are more effective. Quinine is among the least effective of the cinchona antimalarials. Cinchonine and cinchonidine probably owe their high activity partly to the fact that they are not metabolized by the "quinine oxidase" of the liver. Saturation of the double bond to produce dihydro-derivatives probably increases the antimalarial activity of the *l*-isomers.

chow containing 13 micrograms of iodine. The rats, of mixed sexes, weighed 275 grams on the average. After twenty-three days of such treatment, each group of animals was changed to a diet containing the same basic dog chow plus another drug, or iodide, or a combination of the two. At appropriate intervals thereafter, individual animals were sacrificed over the course of three weeks.

The various groups of animals studied are listed in the following description

Fig. 1. Schematic Summary of the Experiments Reported Herewith, Based upon the Weight of Fresh Thyroid

The preliminary period of thiouracil treatment has been broken to save space. Furthermore, the right-hand portion of the diagram has been distorted to avoid crowding. Actually the solid portions of the lines should be horizontal at the level of the respective means. These solid lines statistically all have slopes which are indistinguishable from zero and from each other's slope.

of the experimental procedure. From each rat the thyroid was removed and its iodine constituents studied. In addition, in selected instances, the pituitary and the circulating plasma "hormonal" iodine were also studied. In this way the respective effects of the several drugs upon thyroidal iodine could be determined. An important feature of the present study is that heavy dosage was employed. In this way borderline effects would be avoided.

The experimental plan can be visualized readily from fig. 1.

EXPERIMENTAL PROCEDURE. The animals were divided into seven experimental groups as shown in the following schedule. Each animal consumed approximately 15 grams daily

GOITROGENIC AGENTS AND THYROIDAL IODINE: THEIR PHARMACODYNAMIC INTERPLAY UPON THYROID FUNCTION[1]

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INTRODUCTION

Recent work on sulfanilamide derivatives (1) and thiourea substitution products has stimulated renewed interest in the internal mechanisms of thyroid physiology. Astwood and his co-workers (2) after studying several hundred chemical compounds, have reported to date none with a greater therapeutic index than thiouracil. Likewise, McGinty and Bywater (3) are studying in detail many other derivatives, as indeed are several other laboratories (4). The mechanism of action still remains obscure and merits further study: because such investigation will not only contribute towards fundamental thyroid physiology but will also elucidate the mechanism of action of these compounds when used as drugs. It is the purpose of this communication to report, first, upon the effect of thiouracil and thiocyanate on the nature of iodine within the thyroid gland; and secondly, upon the interaction of iodide with these goitrogenic agents.[3]

Several contributions to this subject have already appeared, notably by Astwood and Bissell (5) and by Rawson, Tannheimer and Peacock (6). Although such work has contributed materially to an understanding of the action of the goitrogenic drugs, two technical difficulties have prevented altogether satisfactory pursuance of the problem. The first of these difficulties was the lack of methods adequate for the analysis *in a single gland* of a small animal, of the various iodine sub-fractions which represent chemical stages in the natural biosynthetic sequence. The second difficulty, encountered with the use of radio-iodine, was the danger of an "exchange reaction" (7), which would obscure the partition of iodine among the several chemical combinations thereof. The development of catalytic methods (8, 9) now permits a more satisfactory study of iodine metabolism by describing the partition of iodine-containing fractions in the thyroid of a single mouse or rat.

EXPERIMENTAL PLAN. In the major portion of this work the experimental animal selected was the adult white albino rat of the Mendel strain. The thyroids of these animals were depleted of iodine by administering approximately 150 mg. of thiouracil daily, mixed with approximately 15 grams of standard dog

chow containing 13 micrograms of iodine. The rats, of mixed sexes, weighed 275 grams on the average. After twenty-three days of such treatment, each group of animals was changed to a diet containing the same basic dog chow plus another drug, or iodide, or a combination of the two. At appropriate intervals thereafter, individual animals were sacrificed over the course of three weeks.

The various groups of animals studied are listed in the following description

FIG 1 SCHEMATIC SUMMARY OF THE EXPERIMENTS REPORTED HEREWITH, BASED UPON THE WEIGHT OF FRESH THYROID

The preliminary period of thiouracil treatment has been broken to save space Furthermore, the right-hand portion of the diagram has been distorted to avoid crowding. Actually the solid portions of the lines should be horizontal at the level of the respective means. These solid lines statistically all have slopes which are indistinguishable from zero and from each other's slope

of the experimental procedure. From each rat the thyroid was removed and its iodine constituents studied. In addition, in selected instances, the pituitary and the circulating plasma "hormonal" iodine were also studied. In this way the respective effects of the several drugs upon thyroidal iodine could be determined. An important feature of the present study is that heavy dosage was employed. In this way borderline effects would be avoided.

The experimental plan can be visualized readily from fig. 1.

EXPERIMENTAL PROCEDURE. The animals were divided into seven experimental groups as shown in the following schedule Each animal consumed approximately 15 grams daily

of a standard dog chow containing 13 micrograms of iodine. To each diet was added a drug or combination of drugs as indicated for nn idealized 250-gram rat:

GROUP OF ANIMALS	DESIGNATION IN TEXT	PRELIMINARY PERIOD OF 23 DAYS	TEST PERIOD OF 23 TO 25 DAYS
F	"thiouracil"	Thiouracil, 150 mg.	Thiouracil, 160 mg.
G	"KI plus thiouracil"	Thiouracil, 150 mg.	Thiouracil, 160 mg. plus KI, 135 mg.
D	"KCNS after thiouracil"	Thiouracil, 155 mg.	Potassium thiocyanate, 324 mg.
E	"KCNS plus KI"	Thiouracil, 155 mg.	KCNS, 324 mg. plus KI, 135 mg.
B	"chow after thiouracil"	Thiouracil, 160 mg.	16 grams of dog chow containing 13 μg. of iodine
C	"KI after thiouracil"	Thiouracil, 145 mg	Dog chow plus KI, 135 mg.
A	"control"	Dog chow only, 14.3 grams, containing 13 μg. of iodine	Dog chow only, 17 grams

At intervals of about two days the animals were sacrificed *seriatim*. Each animal was killed rapidly with ether if necessary, after removal of heart's blood by cardiac puncture. Thereupon, the thyroid and pituitary were excised rapidly. The thyroid tissue was weighed and plunged into five per cent aqueous ammonium hydroxide. With the help of a micro-homogenizer the gland was rapidly dispersed throughout the medium until homogeneous. Thereafter chemical analyses were made of total iodine or appropriate fractions thereof.

ANATOMICAL METHODS. In selected animals one thyroid lobe was reserved for histological examination. After weighing, this was fixed in ten per cent formalin preparatory to histological sectioning. The histological sections were stained with hematoxylin and eosin.

At the time each rat was killed for the thyroid study, its pituitary was immediately removed and carefully freed from dura mater. The gland was fixed in Bouin's fluid and subsequently embedded in paraffin. The sections were cut at 4 micra and stained with acid fuchsin. They were counterstained with Mallory's aniline blue, orange G, and phosphomolybdic acid.

CHEMICAL METHODS. The determination of total iodine was made upon the ashed suspension after appropriate sampling. Chemical sub-fractions were determined by a modification of the method of Leland and Foster (10), utilizing the differential partition between aqueous and butyl-alcohol media with change of acidity. Ashing was accomplished in a small electric furnace, equipped with a pyrometer. The temperature was maintained for three hours between 600°C. and 650°C. A detailed description of this procedure has been published elsewhere (9). Iodine was determined by the catalytic reduction of cerie ions as described by Sappington, Halperin and Salter (8).

EXPERIMENTAL FINDINGS. *Weight of fresh thyroid.* The thyroids in the respective groups were subjected both to histological examination and to chemical analysis. In all groups except the control group A, there was marked hypertrophy of the gland, as indicated in Table I. There was considerable scattering within groups. The mean weights for all the groups are indicated by the following values reduced to 100 grams of rat:

For group A (control) 6.1 mg.; group B (chow after thiouracil) 14.1 mg.;

group C (KI after thiouracil) 16.0 mg.; group D (KCNS after thiouracil) 21.3 mg.; group E (KCNS plus KI) 15.1 mg.; group F (thiouracil) 30.6 mg.; group G (thiouracil plus KI) 39.8 mg.

(a) *Anatomical findings.* Observations on histological sections of thyroids stained with hematoxylin-eosin. Histological examination of the thyroid glands in these groups of animals revealed the following points:

Those animals receiving thiouracil alone (Group F) showed a marked hyperplasia of the thyroid epithelium. In many cases the acinar configuration of the thyroid was lost completely, and in no case was any colloid visible. In those animals receiving potassium iodide along with the thiouracil (Group G), the degree of hyperplasia of the thyroid epithelium was not as marked, although considerable hyperplasia existed. In the majority of such cases the acinar lumina were distinctly present, and they usually contained a small amount of granular eosinophilic material. In only one animal was there actual colloid present, and in this animal the acinar epithelium was only slightly hypertrophied.

In the group of animals in which the thiouracil was followed by chow alone (Group B), the hyperplasia of the thyroid gradually diminished and colloid gradually reappeared; until by the eighteenth day after cessation of thiouracil the gland resembled that of a normal rat. When the chow diet was supplemented with potassium iodide there was no significant difference in the rate at which hyperplasia declined.

The addition of thiocyanate to the chow following thiouracil therapy (Group D) prolonged the period of hyperplasia. When potassium iodide and thiocyanate were combined (Group E), some of the thyroids exhibited a very irregular picture. In certain regions there were acini with flat epithelium and abundant colloid. In other regions there were acini with hypertrophied epithelium and no colloid. Many animals in this group, however, showed almost no evidence of hypertrophy, i.e., the epithelium was low cubiodal and the acini were well filled with eosinophilic colloid.

In summary, it will be noted that these findings compare harmoniously with the gross weights recorded in Table I.

Observations on histological sections of pituitaries stained with acid fuchsin and Mallory's aniline blue. The anterior lobes of the pituitaries were examined for changes in relation to the various combinations of drugs following the preliminary treatment with thiouracil. The following observations were made:

In those animals receiving thiouracil alone (Group F), the drug produced many basophils and caused eosinophils nearly to disappear. The basophils increased both in number and in size. Most of them exhibited typical vacuolization and blue-staining hyaline material within the vacuoles. The chromophobes were somewhat increased in number. These changes are characteristic of those described by Severinghaus, Smelser, and Clark (11), Zeckwer et al. (12) and Guyer and Claus (13) following complete thyroidectomy in the rat. Similar changes were found by Mackenzie and Mackenzie (1) and Griesbach and Purves (14) following treatment with a goitrogenic agent. The addition of potassium iodide had little effect on the picture, although a few faintly acidophilic granules appeared in some of the chromophobes.

TABLE I

Effect of iodide combined with goitrogenic agent
(Mendel strain rats, mixed sexes)

PURPOSE OF EXPERIMENT	TREATMENT	DAYS AFTER PRELIMINARY THREE WEEKS ON THIOURACIL	AMOUNT EATEN PER DAY BY INDIVIDUAL RAT (grams)	WEIGHT OF RAT Before experiment (grams)	WEIGHT OF RAT After experiment (grams)	WEIGHT OF FRESH THYROID PER 100 GRAMS OF FINAL BODY WEIGHT (mgm per 100 grams)	CONCENTRATION OF IODINE IN FRESH THYROID (mgm %)	TOTAL THYROID IODINE IN MG PER 100 GM BODY WEIGHT (µg per 100 gm)
KI effect on thiouracil	Group Γ — Thiouracil in ground dog chow	2	19		200	11 0	0	0 0
		5	13	238	242	18 3	0	0 0
		6	18	260	180	25 8	0	0 0
		7	16	278	265	11 3	0	0 0
		8	14	380	240	31 2	4 4	0 9
		5	19	265	218	21 3		
		12	15	230	238	23 6	0	0 0
		12	19	410	120	21 7	0	0 0
		11	10	300	340	39 7	0	0 0
		10	19		300	23 8	0	0 0
		18	18	300	260	41 2	0	0 0
		21	11	300	280	33 1	0	0 0
Average			10	296	208	30 6	0 0	0 0
	Group G — Thiouracil and KI in ground dog chow	11	14	340	320	37 6		
		12	11	360	280	26 7	12 0	2 6
		14	18	500	440	29 3	17 5	5 2
		16	20	380	280	49 6	7 7	3 8
		18	10	300	280	51 2	11 4	6 9
		20	13		310	52 8	5 8	3 2
		22	15	380	340	29 1	17 8	5 1
		25	16	260	280	39 6	15 9	6 3
Average			10	360	320	39 8	12 6	4 6

Group E. KCNS and KI in Ground dog chow

	Period I	Period II					
2	17	14	320	320	12.6	13.0	1.6
4	15	20	200	231	20.6	3.1	0.6
6	14	17	360	325	19.1	3.6	0.7
8	18	17	280	292	13.2	5.6	1.0
10	15	15	398	346	11.9	16.0	1.9
11	15	16	400	340	14.5	10.8	1.4
12	9	12	260	280	16.5	6.8	1.1
14	17	17	300	280	11.4	12.4	1.4
16	14	15	360	310	15.1	3.4	0.5
18	18	20	320	300	11.2	23.5	2.6
20	15	14	320	300	18.4	0.0	0.0
25	14	17	320	300	18.1	12.8	1.4
Average	15	16	320	300	15.1	9.8	1.2

Group C. KI in ground dog chow

	Period I	Period II					
KI effect	2	14	17	340	324	15.2	10.6	1.6
	6	12	19	210	222	10.9	11.6	1.3
	8	17	20	210	252	15.2	6.6	0.8
	10	17	19	440	450	7.1	2.5	0.2

KI effect on KCNS — Group D. KCNS in ground dog chow

	Period I	Period II					
2	14	9	250	244	21.6	7.1	1.5
5	12	17	211	232	17.2	6.8	1.2
7	15	19	338	300	11.2	25.6	2.9
8	16	19	360	310	34.5	0.0	0.0
8.5	12	16	266	223	17.2	-4.2	0.7
10	11	20	250	200	29.2	0.5	0.2
12	18	22	278	230	12.9	15.7	2.0
12	17	19	120	270	15.0	5.0	0.8
11	12	10	270	280	17.5	0.0	0.0
16	16	19	360	360	24.5	0.0	0.0
18	13	19	250	300	18.3	14.0	2.5
21	11	18	300	300	26.4	0.0	0.0
22	13	16	300	280	34.3	0.0	0.0
Average	11	17	301	271	21.3	5.0	0.8

Group B. Ground dog chow

	Period I	Period II					
KI effect	2	15	15	250	215	11.7	23.1	2.7
	5	16	16	200	267	13.1	15.6	2.1
	7	13	12	252	238	9.3	30.5	3.8
	8	20	20	340	250	24.2	0.6	0.1
	8.5	16	12	268	254	10.7	10.6	1.1
	10	15	19	300	330	18.8	4.9	0.5

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TABLE I—Continued

PURPOSE OF EXPERIMENT	TREATMENT	DAYS AFTER PRELIMINARY THREE WEEKS ON THIOURACIL	AMOUNT EATEN PER DAY BY INDIVIDUAL RAT Period I	AMOUNT EATEN PER DAY BY INDIVIDUAL RAT Period II	WEIGHT OF RAT Before experiment	WEIGHT OF RAT After experiment	WEIGHT OF FRESH THYROID PER 100 GRAMS OF FINAL BODY WEIGHT	CONCENTRATION OF IODINE IN FRESH THYROID	TOTAL THYROID IODINE IN MG PER 100 GM BODY WEIGHT
			gms	gms	grams	grams	mgm per 100 grams	mg %	µg per 100 gm
	Group B—Continued								
Group B Ground dog chow—Cont		12	15	18	268	278	9 5	9 1	0 8
		12	17	20	320	360	24 6	33 6	8 3
		11	19	20	330	280	11 5	10 1	1 5
		16	15	20	290	300	18 9	3 3	0 6
		18	12	13	330	260	9 8	9 9	1 5
		21	13	18	330	360	11 8	0 0	0 0
		22	11	20	160	380	9 8	41 0	4 4
Average			15	17	310	292	11 1	15 8	2 5
	Group C—Continued								
Group C KI in ground dog chow—Cont		12	13	18	300	210	14 0	9 4	2 0
		14	13	17	440	340	15 1	23 0	3 1
		18	8	6	260	190	22 8	8 9	2 0
		20	12	15	360	330	17 4	8 5	2 7
		22	13	12	280	240	12 8	16 1	2 1
		25	11	17	300	280	13.7	25 0	2 4
Average			13	16	314	284	16 0	11 3	2 4

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Group A

Control	Rat No.	17	309		61	65 4	43
Group 1 — Ground dog chow throughout	2	13	270		51	76	41
	2	16	300		51	58	26
	1	16	260		72	93	67
	5	13	278		13	100	78
	6	16	323		57	48	80
	7	13	218		52	34	01
	8	16	286		75	51	88
	9	20	300		97	7	46
	9	11	295		55	72	11
Group A X B — No preliminary thiouracil	9.5	10	232		72	45	77
Rats ate ground dog chow throughout experiment	10	20	300		6.0	36	35
	12	11	292		60	157	59
	12	20	300		10	10	61
	11	20	380		90	55	62
	10	20	500		41	36	77
	18	20	300		68	41	33
	21	20	320		89	74	77
	22	20	380		74	55	41
Average		17	309		6 1	65 4	43

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When thiouracil was stopped and chow (alone) resumed (Group B), the baso-phils disappeared gradually and the eosinophils appeared rapidly. On the eighth day after stopping thiouracil there was a complete return of acidophilic granules but the basophils were still increased in size and number. Thereafter, up to the twenty-second day, the number and size of the basophils gradually declined, as did the extent of vacuolization. The addition of potassium iodide to the chow did not significantly alter the return to normal conditions.

When the thiouracil diet was exchanged for thiocyanate (Group D), the trans-formation toward the normal occurred only gradually. On the eighth day the acidophilic granules were still markedly depleted, but had returned to normal on the sixteenth day of thiocyanate treatment. Throughout this treatment, however, the basophils continued to be increased in size and number and to show a high degree of vacuolization. When the thiocyanate was supplemented with potassium iodide (Group E) no significant effect on the previous picture was noted. On the twelfth day of this combined treatment the acidophils had be-come normal, but vacuolized basophils were prominent. In fact, the basophils continued to resemble those of group D, just described, throughout the combined treatment.

In summary, these histological findings harmonize well with the observations on the gross weights ot the thyroids, as given in Table I.

(b) *Chemical findings.*   The results of the iodine analyses in the various groups are summarized in Table I.   It will be observed that the effect of time was so slight as to be obscured by the biological scattering.   In other words, the storage of fresh iodine occurred early and reached a maximum soon after additional iodide treatment was begun.   Progressive increase in hyperplasia, however, may have favored somewhat the total iodine so accumulated.

ANALYSIS OF EXPERIMENTAL RESULTS.   A troublesome feature of the obser-vations collected in Table I is the marked degree of biological scattering, which becomes evident when one studies each of the 76 animals individually instead of lumping them into groups as has frequently been done in other reports.   This scattering is especially annoying during the early part of the test period when multiple variables such as the rate of hyperplasia, degree of hyperplasia and rate of iodine storage combined to fog the picture.   We are indebted to Miss Barbara L. Bartels for many of the statistical computations cited in this paper.   These have been condensed in the following analysis.

Weight of fresh thyroid.   As illustrated in Figure 1, iodide (Group G) did not prevent the continuous increase in goitrous growth evoked by thiouracil (Group F).   The respective regression coefficients, b = 24 and 27, differed significantly (P < 0.01) from the nearly horizontal slopes of control Group A (b = 2.03) and of Group B "chow after thiouracil" (b = 0.16).   During the latter half of the test period (after 11 days) the means of Groups F and G were distinctly higher than the homologous mean for Group B (chow after thiouracil).   Indeed, P = 0.005.

For thiocyanate the situation is less obvious, but it is highly probable that the goiters increased in size and that iodide prevented this effect.   In the early

days of the test period, random scattering obscured the effect of the drug.   After the eleventh day, however, the mean level attained by the "thiocyanate" group (21.3 mg. per 100 grams of rat) differed significantly from similar means for groups B (chow after thiouracil) and E (KCNS plus KI), i.e., from 14.1 and 15.1 mg., respectively, per 100 grams of rat.

Total thyroid iodine.   When thiouracil was stopped after three weeks of heavy dosage, the test thyroids did not recover completely.   The average total iodine in the glands of control animals (Group A, mean = 4.3) and Group B (chow after thiouracil, mean = 2.5) exceeded that in the test animals.   When iodide was administered with thiouracil (Group G), however, the storage of thyroid iodine was even higher (mean = 4.6) than in the untreated controls.   This exceptionally high level was quite significant ($P = 0.02$) when compared with the "chow" (Group B) animals.

For thiocyanate the case is less clear.   Although iodide probably inhibits the hyperplasia produced by thiocyanate, it does not materially increase the concomitant trapping of iodine.   The mean values of the two thiocyanate groups (D and E, 0.8 and 1.2) do not differ significantly from the mean of "chow" Group B ($P = 0.35$).

Concentration of thyroid iodine.   In three weeks time after stopping thiouracil, the glands did not recover their full ability to trap iodine.   The mean concentration (after 11 days) for control Group A was 65.4 mg. per cent as against 15.8 for "chow" Group B and 5.0 for Group D (KCNS after thiouracil).   However, iodide in high doses nullified, to some extent, the continuing presence of thiouracil.   Whereas the mean for the thiouracil Group F was 0.0, that for G was significantly greater (12.6; $P = 0.001$) and not significantly different from the "chow" Group B.

The effect of thiocyanate, however, was trivial after preliminary hyperplasia from thiouracil.   The mean of the thiocyanate-treated Group D (5.0) is barely distinguishable from the "chow" Group B ($P = 0.05$).   Furthermore, it is not clear that the addition of KI to KCNS (Group E, 9.8) significantly altered the storage of iodine in the gland.

Nature of iodine bound under thiouracil.   Several years ago Salter (15) suggested that the iodine compounds from the thyroid be catalogued under three headings as follows:

(1) "I" iodine, consisting of (inorganic) iodide.
(2) "D" iodine, consisting of compounds resembling diiodotyrosine in their solubility and metabolic behavior.
(3) "T" iodine, consisting of compounds resembling thyroxine.
(4) The combination of "D" and "T" fractions was designated as "P" iodine, because it was bound to precipitated proteins.   It was shown (Salter et al.) (16) that in the analysis of blood serum, it was sufficient in most clinical cases to determine merely the "P" combination as an index of the "hormonal" iodine.

In the study of biosynthesis within the gland, these categories have served to orient further study and to harmonize terminology.   The present work brings

up one further concept, namely, that the first category can be subdivided. It has long been puzzling how the thyroid can trap iodide in high concentration and withold it from tissue fluid which is low in iodide. The present experiments indicate that a loose combination may occur between iodide and a thyroid colloidal system:
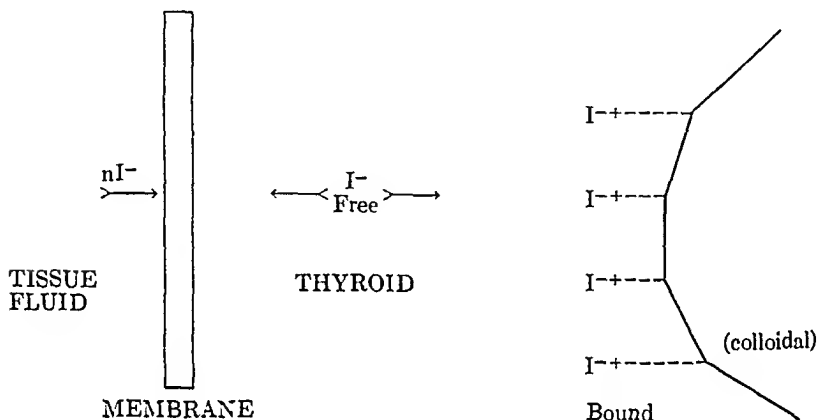
For most purposes, it will not be necessary to subdivide the "I" fraction into sub-fractions Free Iodine, I_F, and Bound Iodine, I_B. In the present case, however, this schema is useful in explaining why Group G animals (treated both with thiouracil and potassium iodide concomitantly) stored so much iodide, in contrast to the analogous experiment with thiocyanate. A further discussion of the I_B complex will appear subsequently (17). At the moment, however, it can be stated that in Group G glands, the iodine is almost wholly in the "I" fraction, and that a major portion of it is in the I_B sub-fraction.

Characteristic analyses upon male rats of the Wistar strain appear in Table II.

Discussion. These results confirm the suspicion hinted at by certain authors (5) that thiouracil can exert a lingering damage to the iodine-trapping function of the thyroid. This hangover appears not to involve the synthesis of thyroid protein to any marked degree. In other words the metabolism of desiodo-thyroglobulin proceeds independently of iodine metabolism

Other authors (18) with a lower dosage of iodide, have found that such doses of iodide counteract the effect of thiocyanate but *not* that of thiouracil. In the present observations the effects of both drugs probably were influenced by iodide; but in a different way. Under thiouracil the hyperplasia was not influenced, but iodine-trapping was. The reverse was true in the case of thiocyanate. It is interesting that iodide in large doses, i.e., 540,000 micrograms per kilo per day, resulted in iodine storage no better than 50 micrograms per kilo per day. Obviously this effect is related to the whole question of the action of iodides in clinical hyperthyroidism. In that disease it has long been known (19) that small doses of iodide aggravate the condition whereas large doses ameliorate it.

TABLE II

Partition of thyroid iodine in presence of goitrogens

(Male.Wistar albino rats)

GROUP	RAT NO.	DAY OF SEC-OND PERIOD	WEIGHT OF RAT	"I" FRAC-TION	"D" FRAC-TION	"I" FRAC-TION	CALCU-LATED TOTAL THY-ROID IODINE	FINAL WEIGHT OF FRESH THY-ROID	CALCU-LATED PERCENT-AGE TOTAL IODINE IN FRESH THYROID	REMARKS
			grams	µg.	µg.	µg.	µg.	mg.	mg. %	
Normal con-trols	13		434	0.3	5.4†	3.2†	8.6*	28	32	
	14		414	0.9	7.4	1.1	9.4	28	33	Combined data on 2 animals
	15		404	0.4	4.8	0.2	5.3	21	26	
	16		400	0.4	2.4	0.3	3.2	22	15	
Avg.................			413	0.5	5.0	1.2	6.6	25	27	
Thiouracil alone, Group F			296†	[0.0]†	[0.0]†	[0.0]†	0.0*		0.0	Data according to Table I
Chow after thiouracil, Group B	16	7	300	0.4	4.3	0.1	4.7	48	9.9	Note accumula-tion of "D" fraction
	17	10	340	0.2	1.0	0.2	1.4	46	3.1	
	18	11	375	0.9	3.3	0.7	4.9	50	10.0	
	19	14	388	0.4	6.3	0.6	7.2	43	16.8	
	20	30	356	0.4	2.1	0.2	2.7	38	7.1	
	21	35	400	[0.0]	2.0	0.3	2.3	32	7.3	
Avg.........			360	0.4	3.2	0.4	4.0	43	9.3	
Thiouracil plus KI, Group G	16	7	340	1.4	[0.0]	0.1	1.5	48	3.0	Note accumula-tion of "I" fraction
	17	10	350	5.0	0.5	[0.0]	5.3	57	10.3	
	18	11	354	4.7	1.8	0.2	6.7	64	10.4	
	19	14	252	4.0	0.8	1.6	6.4	96	6.7	
Avg........			324	3.8	1.0	0.6	5.4	66	8.2	
KI plus KSCN, Group E	16	7	314	0.3	0.4	0.1	0.8	46	1.7	Note low "I" fraction
	17	10	254	0.5	1.0	0.4	1.8	46	3.9	
	18	11	320	0.5	2.3	0.2	3.1	50	6.1	
	19	14	250	0.2	2.7	0.5	3.4	28	12.2	
	20	30	314	0.6	1.0	0.2	1.8	38	4.7	
	21	35	220	[0.0]	1.9	0.7	2.6	28	9.1	
Avg.....			279	0.4	1.5	0.4	2.3	39	5.8	

* Actual determination.

† Estimated.

Why is there so little recovery in the iodine metabolism of the gland after three weeks without the drug? The indications from the literature (20) and from our own observations are that thiouracil is probably largely excreted within a few days. The possibility exists, therefore, that the drug produces a

semipermanent defect in the enzymic mechanism concerned with the fixation of iodine. Where might this interference occur? It has been stated (5, 6) that under certain circumstances thiouracil prevents the accumulation of iodine. In the presence of large doses of iodide, however, as shown in the "KI plus thiouracil" Group G, this would seem not to be the case; although the stored iodine is not the "D" or "T" type so characteristic of normal thyroglobulin. A similar result was obtained by Schachner, Franklin, and Chaikoff (21), working with surviving slices of thyroid tissue in the presence of thiouracil and potassium iodide, combined.

Such iodine may represent only an unstable "adsorption" combination. Thiouracil does not interfere with the accumulation of this "inorganic" iodine complex, and may even be a partner to its combination. The drug, however, creates a block to the subsequent iodination of tyrosine. Possibly the block is related to oxidation-reduction potentials as described by Lowenstein (22) and by de Robertis (23).

Special attention should be paid to the great biological variation which may be encountered in work with these goitrogens. In the present report this variation is evident both among animals of the same strain (Table I) and between different strains (Table I vs. Table II). Furthermore the range of dosage tolerated by immature animals is probably less than the dose tolerated by older animals. In the present work, only three of the mature animals died spontaneously and, on the average, the others maintained weight and seemed healthy. Therefore heavy dosage was employed in order to avoid borderline effects. By contrast, the following comment kindly contributed by Dr. E. B. Astwood is illuminating:

"The actual data differ considerably from our experience in many respects. A diet of 1% thiouracil in our experience is distinctly toxic and about one third of the animals died with stones in the urinary tract. The highest concentration of potassium thiocyanate which does not markedly retard growth, in our experience, is about 0.25% of the diet; larger amounts are lethal to quite a number of animals. Even 1% potassium iodide distinctly retards growth in young rats. Perhaps some of the differences between your results and ours are due to the toxic dosage—this seems to be particularly likely with potassium thiocyanate. Toxic substances have an effect similar to hypophysectomy and we have found that compounds even more active than thiouracil have practically no effect upon the size of the thyroid gland when given at near-lethal levels.

"Some points of difference between your results in Table I and ours are as follows: Group A agrees well with our values except that our variation is much smaller. We have never seen concentrations of iodine as low as eight of the values shown or as high as 157 mg. per cent in normal rats. Group B and C differ greatly from ours; withdrawal of thiouracil has consistently permitted a return nearly to normal size and concentration of iodine. In our experience the addition of potassium iodide to thiouracil causes a much higher concentration of iodine to accumulate than that shown for Group G. Our average was between 30 and 40 mg. per cent in contrast to your 12.6.

As our animals were young the two sets of data are, of course, not strictly comparable."

SUMMARY

When large doses of thiouracil are administered to rats, a persistent failure to trap iodine results. In part, this deficiency can be overcome by large doses of iodide. In this respect thiocyanate seems to differ from thiouracil both in the qualitative response and in the quantitative aspects of the dosages used. In the presence of either drug large doses of iodide will facilitate the storage of iodine; but conversion of iodide to diiodotyrosine (and incidentally to thyroxine) is prevented.

The synthesis of *uniodinated* thyroid protein and cellular hyperplasia can proceed unimpaired despite heavy dosage of thiouracil. Hyperplasia under thiocyanate, however, is nullified by iodide.

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A CONTRIBUTION TO THE PHARMACOLOGY OF 5-NITRO-2-FURALDEHYDE SEMICARBAZONE[1]

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Comparatively little information is available concerning the pharmacologic properties of furan and its derivatives. Gilman and Pickens (1) found several furan derivatives to elicit local anesthetic properties. Koch and Cahan (2) after a study of the five membered ring, furan, on several species concluded, that while furan produced some anesthetic and analgesic properties its inherent toxicity eliminated it from consideration in therapeutics. These workers classified furan as a protoplasmic poison. Recently Dodd and Stillman (3) prepared forty-two furan derivatives and studied their bacteriostatic properties *in vitro* against several kinds of pathogenic organisms. They concluded that bacteriostatic potency was dependent upon a nitro group on the ring and their active furan derivatives contained a nitro group in position "5". The most promising of the furan derivatives of this series was 5-nitro-2-furaldehyde semicarbazone. Its relationship to furan is shown by the following formulas.
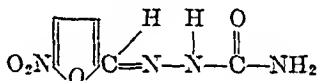
Furan   5-Nitro-2-furaldehyde semicarbazone
(Furacin)

The bacteriostatic activity of this compound is manifested *in vitro* at high dilutions.

Dodd (4) found this compound promising as a chemotherapeutic agent in laboratory animals.

Snyder et al. (5) reported the successful use of this compound in the local treatment of infected wounds.

Owing to the promising properties of 5-nitro-2-furaldehyde semicarbazone, a pharmacologic study of its properties seemed warranted.

Physical properties. 5-Nitro-2-furaldehyde semicarbazone occurs in bright yellow crystals which decompose between 227° and 241° depending upon the speed of heating. One gram dissolves in about 5 liters of water. It is less soluble in ether, but dissolves in alcohol 1:740 and in propylene glycol 1:350.

Blood pressure and respiration studies. Blood pressure studies were conducted on 3 dogs under ether anesthesia, 3 cats under pentobarbital sodium anesthesia and 2 *Macacus rhesus* monkeys under pentobarbital sodium anesthesia. Carotid

blood pressures were recorded with a mercury manometer. Injections were made into the saphenous vein.

Saturated solutions of 5-nitro-2-furaldehyde semicarbazone in propylene glycol produced slight, transient rises in blood pressure in the 3 species. Injections were made on the basis of 0.5 cc./kg. No changes in blood pressure were observed that could not be produced by the injection of the solvent alone. Aqueous suspensions, 0.5 and 1 per cent in 6 per cent acacia were injected on the basis of 0.5 to 1 cc./kg. These injections produced depressor responses upon repeated injection. However, these changes were produced by similar injections of talc and hence considered to be due to the injection of insoluble particles into the circulation. After injections of 5-nitro-2-furaldehyde semicarbazone, the blood vessels responded in their characteristic manner to epinephrine and histamine injections respectively.

No significant changes in rate or amplitude of respiration were recorded that could not be attributed to the vascular effects of the injection of insoluble particles.

Electrocardiographic studies. In 2 of the dogs and each of the 2 monkeys used in the foregoing studies, electrocardiogram Leads I, II and III were taken under anesthesia and shortly after the injections of 0.5 per cent suspensions of 5-nitro-2-furaldehyde semicarbazone. There appeared to be no significant difference in the regularity or form of the tracings in any animal in any Lead.

Perfusion frog's heart. 5-nitro-2-furaldehyde semicarbazone was agitated with Locke-Ringer's solution until saturated. This solution was perfused through the frog's heart *in situ.* Seven experiments were conducted on 3 frogs. There appeared to be no change in the heart rate, regularity or amplitude upon perfusion with the saturated solution of the furan derivative.

Perfusion isolated mammalian heart. The rabbit's heart was extirpated after killing the animal by cerebral contusion. The defibrinated blood was mixed with Locke-Ringer's solution and perfused through the aorta. Tracings were made of the heart's beat. At intervals, 5 cc. volumes of saturated solution of 5-nitro-2-furaldehyde semicarbazone in Locke-Ringer's solution were introduced into the perfusion fluid. Four experiments were conducted and in each instance, the effect upon the rabbit's heart coincided with the results obtained on the amphibian heart *in situ,* namely, there occurred no change in rate, regularity or amplitude of beat.

Perfusion of the leg vessels of the frog. In typical Trendelenberg perfusion experiments of the leg vessels of the frog, saturated solutions of 5-nitro-2-furaldehyde semicarbazone in Locke-Ringer's solutions, neither increased nor decreased the number of drops perfusing through the abdominal vein during a constant time period of 30 seconds. There were 10 experiments carried out on 6 frogs.

Cytochrome oxidase and tissue dehydrogenases. Fresh minced suspension of rabbit's brain served as a source of cytochrome oxidase and tissue dehydrogenases.

The following procedure was used to study the effect on the cytochrome

oxidase. Two cc. samples of the brain suspension were placed in three 15 cc. test tubes. One sample was boiled for 1 minute. To another was added 1 cc. of normal salt solution and to the third test tube 1 cc. of normal salt solution previously saturated with 5-nitro-2-furaldehyde semicarbazone. To each of the 3 tubes was added 0.5 cc. of freshly prepared "Nadi" reagent (indophenol reaction for cytochrome oxidase). The tubes were shaken gently and the time required for the development of the purple color noted. The time required was 120 seconds± 10 seconds for the control and the furan derivative treated tissues, indicating that 5-nitro-2-furaldehyde semicarbazone, under these conditions, did not affect the cytochrome oxidase. The boiled minced tissues developed no color. The experiment was repeated 6 times.

Typical Thunberg, methylene blue-reduction experiments were employed to determine the effect of the furan derivative on the tissue dehydrogenases. One cc. samples of the minced brain were placed in modified Thunberg tubes. To these was added 1 cc. of methylene blue, 1:5000 in M/50 disodium hydrogen phosphate. To one sample, 1 cc. of normal salt solution was added, and to the other 1 cc. of normal salt solution previously saturated with 5-nitro-2-furaldehyde semicarbazone. The tubes were evacuated and placed in a bath at 37°. The time in minutes required for the decolorization of the methylene blue was recorded. There were 6 tests and 6 controls conducted. The average time for the controls was 14 minutes, for the furan treated tubes 31 minutes indicating that this compound retards the activity of the tissue dehydrogenases.

Oxygen uptake (rat). The oxygen uptake of the white rat was determined by the method used by Teitelbaum (6). The animal was fasted 24 hours, placed in the metabolism chamber at 28°C. for 30 minutes to become quiet and equilibrated in an atmosphere of oxygen. The oxygen consumption was measured over a 30 minute period. The animal was removed and given by stomach tube half the LD_{50} dose of 5-nitro-2-furaldehyde semicarbazone. The animal was again placed in the metabolism chamber and 60 minutes allowed to elapse for the absorption of the compound and equilibration. Then the oxygen consumption was measured for 30 minutes as in the control test. There were 6 experiments conducted. There was no significant effect. The trend, however, indicated increased oxygen consumption

Acute toxicity (rat). 5-nitro-2-furaldehyde semicarbazone was suspended in 10 per cent acacia solution and administered to male white rats, 50 to 100 gm. by stomach tube. The number of deaths after 48 hours was observed. Table 1 records these data.

The LD_{50} was calculated by Karber's (7) method using the formula Log $LD_{50} = X_0 - \sum \dfrac{(P_1 + P_2)d}{2}$ in which X_0 is the logarithm of LD_{100}. The second term is the sum of the products. Each product is obtained by multiplying the mean of each successive pair of observed mortalities by the difference between the logarithms of the corresponding doses. The value obtained was 59 mg./100 gm. rat. Animals of the group which died showed premonitory symptoms of

hyperirritability, hyperreflexia, tremors, weakness and convulsive seizures; respiratory arrest appeared to be the ultimate cause of death.

To determine whether or not the hyperreflexia produced by 5-nitro-2-furaldehyde semicarbazone was accompanied by hyperpyrexia, the rectal temperatures of 4 rats were determined. One hundred milligrams per 100 gm. of body weight were administered orally and 3 hours later, when the animals were definitely in the hyperreflexic to convulsive stages of intoxication, the tempera-

TABLE 1

LD_{50} 48 hours (rat)

DOSAGE	NUMBER OF RATS	NUMBER DEAD	PERCENTAGE DEAD
mgm./100 gm.			
10	4	0	0
20	5	1	20
30	15	3	20
40	25	8	32
50	45	19	42
60	40	14	35
70	20	8	40
80	10	8	80
100	4	4	100

TABLE 2

LD_{50} 48 hours (mouse)

DOSAGE	NUMBER OF MICE	NUMBER DEAD	PERCENTAGE DEAD
mg./100 gm.			
100	10	9	90
80	10	10	100
60	32	28	88
40	40	31	77
30	44	27	61
20	30	3	10
10	20	1	5
5	10	0	0

tures were again determined. No significant difference was observed in any of the animals.

Acute toxicity (mouse). These studies were conducted using white mice weighing approximately 15 gm. The data are shown in table 2. The LD_{50} calculated by the foregoing method is 38 mg./100 gm.

Chronic toxicity studies (monkey). Two *Macacus rhesus* monkeys were fed 0.3 gm. of 5-nitro-2-furaldehyde semicarbazone each day for a period of 5 weeks. Weekly weight records were made. A blood picture was made prior to and after the feeding period. Just prior to the termination of the experiment, an electrocardiogram and electroencephalogram were taken on each animal. The

animals were then anesthetized with pentobarbital sodium and a hepatic biopsy and a unilateral nephrectomy performed. The tissues were examined histologically.

During the 5 week period of feeding 5-nitro-2-furaldehyde semicarbazone the monkeys showed no significant weight variations. The electrocardiogram showed no significant difference in regularity or form in any Lead for either animal. Electroencephalograms made on the treated animals before and after sedation with pentobarbital sodium showed no significant variation from untreated monkeys under the same treatment. There was no significant variation in the blood picture of the treated animals.

The histologic findings in the tissues were as follows:

B-186. The section of kidney shows no abnormality. The individual liver cells are pale, vesicular and granular. This condition is generalized with no focal necrosis or lobular concentration. The section from the sternum is entirely cartilagenous and muscular. No bone marrow is present.

TABLE 3

Blood counts (monkeys)

	R B c./cmm.	w.b c./cmm.
A. Prior to feeding		
No. 1 R	6,200,000	16,400
No. 2 S	6,810,000	16,650
B. After feeding		
No. 1 R	7,200,000	16,000
No. 2 S	5,500,000	15,250

B-187. Section from kidney is normal. The sternal marrow appears normal. An adherent lymph node shows a granulomatous and necrotic inflammatory process that resembles tuberculosis. Section from the liver in this animal shows a moderate fatty change that is deposited chiefly in the periphery of the lobules. This may represent storage.

Three additional monkeys were subjected to unilateral nephrectomy under pentobarbital sodium anesthesia. One week after the operation blood counts were made. Each day for 6 consecutive days, each animal was given 0.5 gm. 5-nitro-2-furaldehyde semicarbazone. At the end of the period another blood count was made and the animals sacrificed for histologic study of the liver and kidney.

Table 3A shows the blood counts of the monkeys.

The following histologic findings were present: The sections from these three animals appear identical. There is a scant amount of coagulated fluid in the renal tubules in each. The individual liver cells are pale, granular and swollen. This is generalized without focal concentration. These minor changes are probably of little significance.

Chronic toxicity studies (rat). Young male rats were fed a normal laboratory ration of supplemented Purina fox chow as controls. Groups of 12 animals were fed the same ration to which was added 0.4, 0.2 and 0.1 per cent respectively of 5-nitro-2-furaldehyde semicarbazone. The blood picture was taken prior to and after the experiment. The data are shown in table 4. Weights were recorded biweekly. At the end of a 5 week period 3 animals from each group were sacrificed for histologic study.

TABLE 3 A

Blood counts (monkeys)

	R B C /c.mm	W.B C /c.mm.
A. Prior to drug		
No 1	2,960,000	6,300
No. 2	4,530,000	8,800
No 3	4,441,000	8,750
B After feeding		
No 1	3,430,000	13,300
No. 2	5,100,000	13,750
No. 3	4,870,000	14,550

TABLE 4

Blood counts (rats)

	R B C /c.mm.	W B C /c.mm.
A Prior to feeding		
No. 1	6,670,000	7,250
No. 2	6,670,000	7,750
No 3	6,400,000	7,600
No. 4	6,600,000	7,400
B. After feeding		
No 1 0 1 per cent	7,260,000	16,200
No 2 0 1 per cent	9,100,000	19,600
No 3 0 2 per cent	6,400,000	15,550

The growth curves of these animals are seen in chart 1. These data set forth in chart 1 indicate that rats tolerated 0.1 and 0.2 per cent respectively of 5-nitro-2-furaldehyde semicarbazone in their food but not 0.4 per cent. The animals in the 0.4 per cent group became cachetic and hyperexcitable and most of them died within one week after the beginning of the experiment.

Four of these animals were sacrificed for histologic study. The findings are set forth in the following descriptions: Sections from liver, kidneys and intestines appear similar in these four experimental animals. All the kidneys with the exception of one show coagulated albuminous fluid in the tubules. In 3 of the 4 livers there were small focal cellular and necrotic areas. The intestines appear normal.

Six of the rats receiving 0.1 per cent 5-nitro-2-furaldehyde semicarbazone and 3 which had 0.2 per cent of the compound were sacrificed for histologic study. The findings are set forth in the following descriptions:

One tenth per cent 5-nitro-2-furaldehyde semicarbazone feeding:

1. There is a mild diffuse swelling and granular change in the cords of the liver cells. No focal necrosis. Sections of kidney, intestine and bladder are not significant.

2. Liver normal. No significant change in bladder or intestine.

3. Liver, bladder, intestine and pancreas show no significant deviation from the controls.

4. The liver cells are more swollen and granular than normal. Coagulated
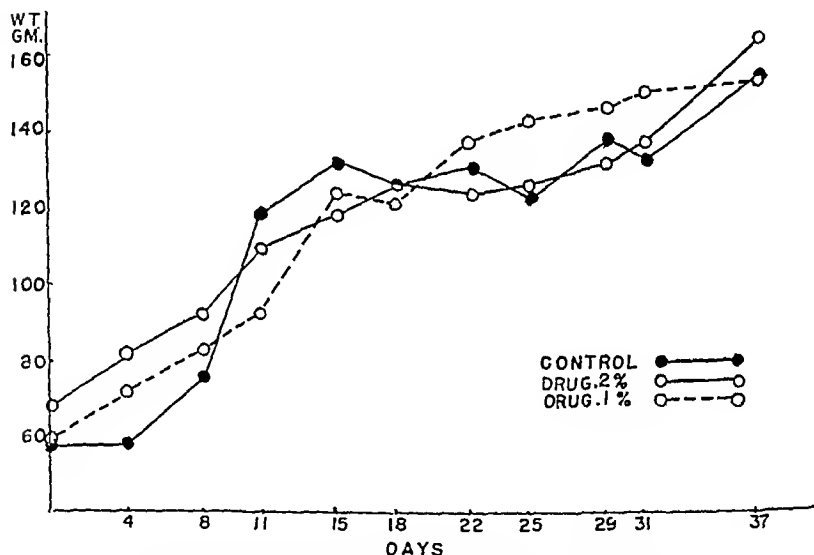
CHART 1. GROWTH CURVES OF RATS RECEIVING 0.1 AND 0.2 PER CENT 5-NITRO-2-FURALDEHYDE SEMICARBAZONE IN THE DIET

fluid is present in some of the renal tubules. The pancreas, intestine and bladder appear normal.

5. The bladder displays edema, swelling and granularity. The kidney, bladder and intestines are not significant.

6. There is mild cloudy swelling of the convoluted tubules in the kidney and the liver cords. The other organs are normal.

Two tenths per cent 5-nitro-2-furaldehyde semicarbazone feeding:

7. There is no significant change in the liver, bladder or intestine. Coagulated fluid is present in the renal tubules.

8. Mild cloudy swelling is present in the liver. Sections from kidney, pancreas, bladder and intestine are not abnormal.

9. There is mild cloudy swelling of the liver but no focal necrosis. The kidney, intestine and bladder show no abnormality.

Preliminary experiments in man. Having established the fact that relatively large amounts of 5-nitro-2-furaldehyde semicarbazone could be ingested or injected into several species of animals with comparatively little injury to the important viscera, one of us (W. E. E. Jr.) ingested 100 mg. with no discernible symptoms following.

Several individuals were given 100 mg. three times a day without symptoms being produced. The dose was increased until 2 to 4 grams were given daily. These doses were tolerated by many individuals asymptomatically. In approximately 20 per cent of the individuals the drug produced nausea. Routine blood and urine chemical findings were not distorted by the ingestion of the drug.

SUMMARY

1. A pharmacodynamic and toxicologic investigation of 5-nitro-2-furaldehyde semicarbazone has been made in several species of animals.

2. In quantities, indicated by bacteriological studies as therapeutic, the compound appears to be tolerated without symptoms. Toxic doses produce hyperexcitability of the central nervous system in the rat.

3. *In vitro*, 5-nitro-2-furaldehyde semicarbazone diminished the activity of tissue dehydrogenases.

4. Preliminary ingestion trials in man have been recorded.

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REACTION OF TISSUE ALDEHYDES WITH CERTAIN DRUGS

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Demonstration of a chemical combination of drugs with one particular component of the cell might serve to shed some light on the problem of the fixation of drugs by cells. Oster and Mulinos (1) have recently reported that tissue aldehydes and various amines react with the resultant formation of Schiff's bases. Since it was suggested at that time that the tissue aldehydes may play an integral role in a detoxification mechanism for amines, it was deemed worthwhile to investigate the reaction of additional amines and to study the behavior of compounds with other groupings prone to form chemical combinations with aldehydes. The selection of these substances was prompted by their being naturally occuring compounds, commonly used drugs or the basic constituents of drugs.

Furthermore, in view of Kohn's recent report (2) on a new aerobic metabolite which is supposedly of aldehydic nature, it was assumed that this "substance B" might well be a tissue aldehyde, citing interaction with amines as evidence.

EXPERIMENTAL. Kidneys of rats of either sex were again chosen as test organs. Frozen sections of fresh rat kidney, 50 micra in thickness, were incubated for 18 hours at 37°C. in N/20 HCl, using 0.85% NaCl as the diluent. This procedure liberates bound tissue aldehydes. These sections were washed in normal saline and incubated for 18 hours at 37°C. in 0.1% solutions of the compounds on test, using M/15 KH_2PO_4 buffer. If necessary, the pH was adjusted to a value of from 3 to 5 by the addition of ascorbic acid. The sections were then stained with fuchsin sulfurous acid (FSA). The depth of the developing purple color was compared with that of the control sections which had been similarly incubated in M/15 KH_2PO_4 buffer without the addition of a drug. The degree of staining of the control sections was given a value of 4 plus. Test sections were designated as negative to 4 plus, according to the stainability of the sections. A 4 plus FSA staining reaction denotes non combination of the liberated tissue aldehydes with the drug and consequent ability to react with the FSA. Negative or 1 plus reactions would indicate complete combining power with the added chemical. All substances were tested on kidney sections from three different rats.

RESULTS. Table 1 summarizes the results obtained with the various substances used. An analysis of the findings reveals the following. Acriflavine hydrochloride, containing two amino groups on the ring, combines readily with the tissue aldehydes at 37°C. 9-Aminoacridine hydrochloride, which was insoluble in phosphate buffer solution, combines with the aldehydes when incubated in water. The combining power of para-amino hippuric acid must be due to its amino group, since hippuric acid alone had no effect. Chloramine T, an amide with sodium and chloride substitutions, had no combinative effect. Dichloroazodicarbonamidine (chloroazodin), with two available amidine groups, also effected no reaction. Penicillin sodium did not show any influence.

A casein hydrolysate, taken as representative of an amino acid mixture, was previously tested with no combining results (1). Knowing that tryptophane combines readily with aldehydes, it was surprising to learn that it did not do so with the tissue aldehydes. On the other hand, cysteine hydrochloride did form a combination with the tissue aldehydes. It seems possible that the

TABLE I

Depth of stain of kidney tissue with FSA after hydrolysis with N/20 HCl for 18 hours at 37°C. and subsequent incubation with each drug in phosphate buffer for 18 hours (pH 3 to 5)

COMPOUND TESTED, 0.1%	FSA REACTION
Acriflavine hydrochloride	−
9-Aminoacridine hydrochloride*	−
p-Amino hippuric acid	+
Hippuric acid	++++
Chloramine T	++++
Chloroazodin	++++
Penicillin sodium	++++
Tryptophane	++++
Cysteine hydrochloride	−
Glutathione	++++
Cystine	++++
Neo-Synephrine	++++
β-Phenylethylamine	−
Thiobarbituric Acid	−
Barbituric Acid	−
Phenobarbital	++++
Pentobarbital	++++
Thiouracil	++++
Caffeine alkaloid	++++
Theobromine sodium and sodium salicylate	++++
Allantoin	++++
Adenine sulfate	++++
Rhodanine	−
Hydantoin	++++
Buffer (control)	++++

* Tested in water. Insoluble in phosphate buffer.

mercaptane group of cysteine reacts with the tissue aldehydes, forming a mercaptal.

$$RCHO + 2\ HSCH_2CHNH_2COOH \rightarrow RCH \begin{matrix} SCH_2CHNH_2COOH \\ \\ SCH_2CHNH_2COOH \end{matrix} + H_2O$$
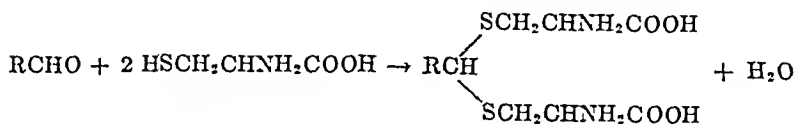
This cysteine-aldehyde end product is not very stable, being easily split by weak acids. However, glutathione, which also contains a free mercaptane group, did not combine, nor did cystine. α-hydroxy-β-methyl-amino-3-hydroxy

ethylbenzene hydrochloride (Neo-Synephrine, Stearns) showed no effect. β-phenylethylamine again exhibited a stain preventing effect, as was reported previously (1).

Kohn (2) claims to have obtained a new metabolite by trichloracetic acid hydrolysis of oxygenated tissue suspensions, a method similar to that of acid fission used in liberating tissue aldehydes from organs. This metabolite is reported to react readily with thiobarbituric acid. We incubated kidney sections with this compound and noted a combination of thiobarbituric acid with the tissue aldehydes. The same was the case with barbituric acid. We found that only such pyrimidines which contain reactive hydrogens on the 5 carbon atom act in such a way. Phenobarbital and pentobarbital were inactive. No combination resulted with thiouracil (Deracil, Lederle) or with purine compounds. A reaction with the tissue aldehydes took place with rhodanine but not with hydantoin. Both compounds are used in amino acid synthesis for their combination with aldehyde groups (3).

$$RCHO + H_2C \begin{array}{c} OC\text{---}NH \\ | \quad\quad | \\ CO \\ | \quad\quad | \\ OC\text{---}NH \end{array} \rightarrow RCH{=}C \begin{array}{c} OC\text{---}NH \\ | \quad\quad | \\ CO \\ | \quad\quad | \\ OC\text{---}NH \end{array} + H_2O$$

Barbituric acid

$$RCHO + H_2C\text{---}S \begin{array}{c} \\ CS \rightarrow \\ \end{array} \quad RCH{=}C\text{---}S \begin{array}{c} \\ CS \rightarrow H_2O \\ \end{array}$$
OC---NH   OC---N

Rhodanine
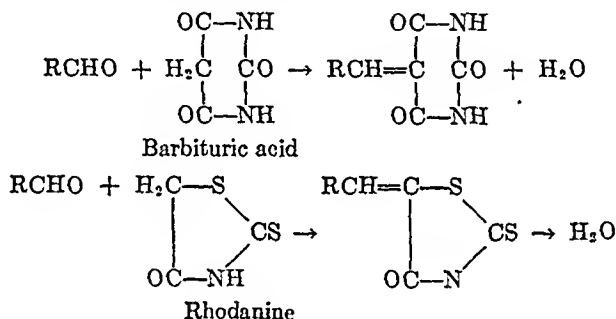
DISCUSSION. The possibility that the tissue aldehydes act as acceptor substances for certain drugs was suggested in a previous publication (1). It was shown that aromatic amines combine readily with the tissue aldehydes. The present investigation demonstrates that the effect extends to cysteine and to barbituric acid. The combination of the latter compound is accomplished by the reaction of the two highly active hydrogens on the 5 carbon atom with the aldehyde group.

The search for an acceptor substance for drugs in the cell is an old one. Tissue aldehydes, due to their intense reactivity and their wide distribution in the various organs, offer themselves for such a possibility, if one assumes that they are in a free state at any given moment during their metabolism.

Recently it was demonstrated by Oster (4, 5) that the metabolism of tissue aldehydes in the rat kidney is influenced by sex hormones, a finding which lends a new significance to these cell constituents Examination of human organs revealed that in some pathological cases the tissue aldehydes disappear completely from certain organs in which they are normally found. Whether this was due to a metabolic derangement or to the action of administered drugs could not be decided Experimental attempts to deplete animals of tissue

aldehydes by injection of para-amino benzoic acid or by sulfa drugs have not been successful thus far (6). However, the in vitro studies presented indicate at least the chemical possibility of such a mechanism. The combination of certain drugs with the tissue aldehydes seems to be one of great specificity under experimental conditions.

In the light of our studies it seems to us highly indicative that the metabolite B described by Kohn is a tissue aldehyde, since the procedure for obtaining it and the chemical combinations which this substance undergoes run parallel to our findings.

SUMMARY

1. Twenty-four compounds were investigated for their possible chemical combination with tissue aldehydes, using frozen sections of rat kidneys as test material.

2. A theory that tissue aldehydes may serve as acceptor substances in the cell for certain drugs is advanced.

3. The similarity of the chemical reactions of tissue aldehydes and Kohn's metabolite B has been shown.

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INDUCTION OF NUTRITIONAL DEFICIENCY BY ORAL ADMINISTRATION OF STREPTOMYCIN

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Nutritional deficiencies may be induced in the rat by the feeding of purified diets containing certain of the sulfonamides (1–2). This effect was ascribed to an inhibition in the synthesis of essential nutrients by the intestinal flora. Gant and co-workers (3) observed a temporary decrease in *E. coli* in the early period of sulfonamide feeding; however, after this time the count returned to normal although the rats declined in weight and gave evidence of developing deficiency disease. These investigators suggested that the new coliform organisms had lost their ability to synthesize the growth factors required by the rat. It is of interest to note that Thompson (4) reported the synthesis of biotin and folic acid by the closely related organism *A. aerogenes*. Miller (5) confirmed the findings of Gant and co-workers and in addition observed lowered levels of biotin, folic acid and pantothenic acid in the feces of rats fed purified diets containing succinylsulfathiazole or phthalylsulfathiazole.

Two antibiotic agents, namely streptothricin and streptomycin, are bacteriostatic both *in vitro* and *in vivo* against a variety of gram-negative organisms. These drugs are poorly absorbed from the intestinal tract as are the relatively insoluble sulfonamides (sulfaguanidine and succinylsulfathiazole). Smith and Robinson (6) working with mice maintained on natural food rations containing streptothricin or streptomycin observed an inhibition of *E. coli* growth which persisted as long as these antibiotics were administered. It seemed possible, therefore, that nutritional deficiencies might be produced by the oral administration of these drugs. Accordingly, weanling male rats were given streptothricin as an adjunct to a purified diet. Unfortunately, due to the relatively high toxicity of this antibiotic the animals given the higher doses succumbed *before the development of a nutritional deficiency could be expected* to take place. Rats receiving smaller sub-lethal doses for several months appeared normal in all respects. The question, whether sterilization of the intestinal tract by agents other than the sulfonamides would produce the same type of nutritional deficiencies, remained therefore unanswered, due to the limited tolerance of the drug. The lower toxicity of streptomycin made it possible to administer higher, chemotherapeutically fully effective doses. Two levels were employed in the diets; one which had been shown previously to eliminate the coliform bacteria in the intestinal tract of mice (6) and the other a dose which afforded maximum toleration. In order to accelerate the rate of depletion and to prevent the possibility of fastness to the drug all animals were given a supplemental oral dose of streptomycin on the first and second days of the test.

EXPERIMENTAL. Weanling male rats were segregated into 6 like groups of 10 animals each and given diets as indicated. The purified ration (H4) had the following composition: casein (Lahco) 24, sucrose 60, salt mix (U.S.P. #1) 4, crisco 10, cod liver oil 2 supplemented with 1 mg. each of thiamine and pyridoxine, 2 mg. riboflavin, 5 mg. inositol, 10 mg. choline chloride per 100 gm. of diet.

The liver containing rations (H4L) were identical with H4 except that 3% of the sucrose was replaced by a like quantity of dried whole liver.[1]

One group given each of the above diets received streptomycin at a level of 2500 units per gm. diet. In addition, a group was given 7500 units per gm. ration as an addendum to diet H4.

All animals receiving streptomycin[2] were dosed by stomach tube with 25,000 additional units of the antibiotic on each of the first 2 days on test. For comparison succinylsulfathiazole was fed to another group as an adjunct to the purified diet at a concentration of 0.75%.

Controls received diets H4 and H4L respectively. It has been established elsewhere and in this laboratory that rats fed dried liver in conjunction with sulfonamide containing rations grow at a normal rate and do not give evidence of nutritional deficiency.

The intake of streptomycin (as calculated from food consumption records) is indicated in table 1. The animals receiving the low level of the drug in conjunction with diets H4

TABLE I

Average daily intake of streptomycin or succinysulfathiazole

WEEKS ON TEST	AVERAGE DAILY INTAKE STREPTOMYCIN—DIET H4 WITH 2500 UNITS/GM.	AVERAGE DAILY INTAKE STREPTOMYCIN—DIET H4L WITH 2500 UNITS/GM.	AVERAGE DAILY INTAKE STREPTOMYCIN—DIET H4 WITH 7500 UNITS/GM.	AVERAGE DAILY INTAKE SUCCINYLSULFATHIAZOLE DIET H4 WITH 0.75%
	units/kg.	*units/kg.*	*units/kg.*	*mg./kg.*
1	364,440	375,000	875,000	1000
2	288,230	307,200	783,050	890
3	223,210	253,900	841,120	740
4	214,280	234,390	766,420	588
5	205,480	198,410	681,810	(All animals dead
6	201,860	159,810	657,890	by 34 days)
7	199,460	185,950	595,670	

and H4L consumed 160,000–275,000 units per kg. per day. The intake on the high level ranged from 600,000 to 875,000 units per kg. per day. These values are exclusive of the 25,000 units administered on each of the first 2 days of the test.

The rats fed the 2 levels of streptomycin in the purified ration grew at a rate that was only slightly below normal for the first 4 weeks of the experiment (fig. 1). No untoward effects other than a transient diarrhea were noted in the animals receiving 160,000–375,000 units of the drug per kg. until after about 6 weeks; at which time the rats became hyperexcitable, vicious and some incoordination in movement was observed. Most of the animals in the group showed some evidence of ventral denudation. The rats receiving the liver, streptomycin containing diet grew at a normal rate but gave evidence of all of

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[1] The liver contained 2.4 μg. of biotin and 31 μg. of folic acid per gm.

[2] The streptomycin employed in these studies was supplied by the Research Laboratories of Merck & Co., Inc. and was equivalent in potency to 165–300 μg. streptomycin base per mgm. of solids (165–300 units per mgm.).
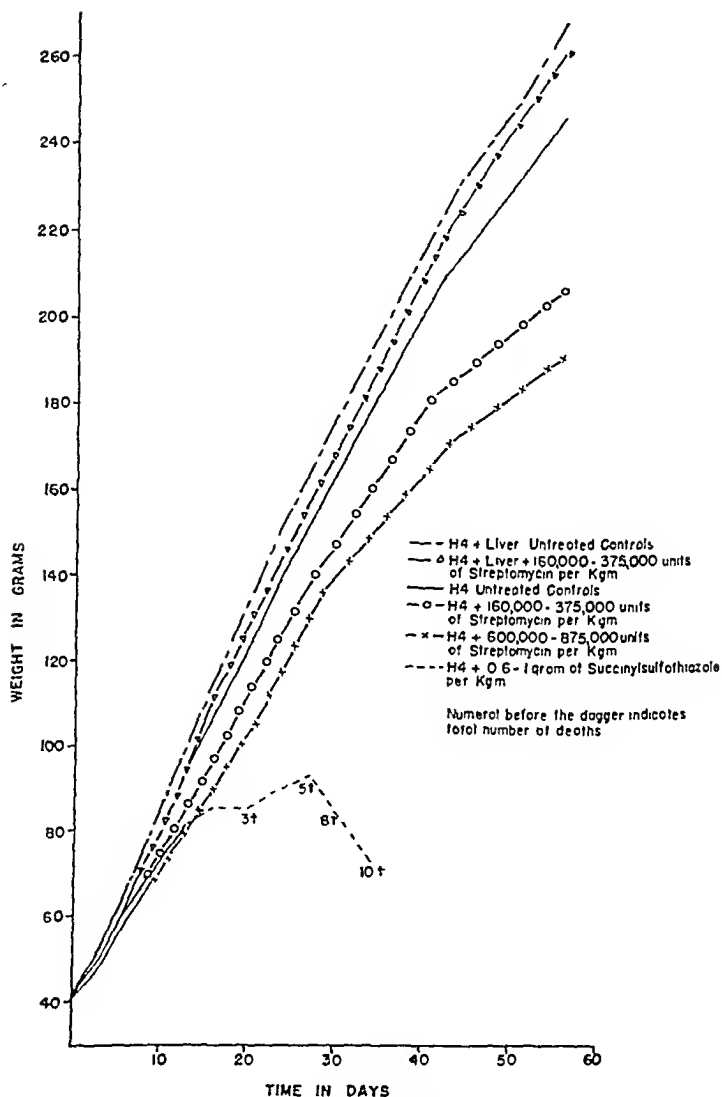
FIG. 1. GROWTH OF MALE RATS RECEIVING STREPTOMYCIN OR SUCCINYLSULFATHIAZOLE
AND THEIR CONTROLS

the above signs except that the alopecia was slight. The animals were sacrificed
after 55 days on test. Gross examination of the tissues at autopsy revealed
no abnormalities.

The rats consuming the larger amount of streptomycin grew at a rate that

was only slightly below normal for the first 4 weeks; after which time a distinct retardation was noted, particularly in 6 of the 10 animals of the group. Toxic manifestations were exaggerated; hyperactivity and hypersensitivity were noted within 24 hours after the beginning of the test. The movements of the animals were rapid, spasmodic and incoordinated. When the animals were handled respiratory depression followed by stimulation and an apparent brady-cardia was observed and subsequent tachycardia. Diarrhea of varying degrees of severity was seen throughout the test period.

Prothrombin determinations were made by the Mushett and Seeler (7) modi-fication of the micro-method of Hoffman and Custer (8). The prothrombin time averaged 16 seconds for both the control and the streptomycin treated groups. Furthermore, the rats did not show leucopenia, granulocytopenia and anemia characteristic of folic acid deficiency (9).

Denudation occurred earlier than with the lower intake of the antibiotic and in some cases involved the entire ventral and posterior dorsal aspects of the trunk. After 55 days on test 4 of the rats showing the most severe alopecia were given 5 μg. of biotin daily subcutaneously. The other animals remained untreated and served as controls. The average weight increments for the en-suing 7 days were as follows: Biotin treated 31 gm., controls 15 gm. The rats receiving biotin gave evidence of new hair growth.

The rats receiving succinylsulfathiazole grew at a normal rate for almost a fortnight; after which they either failed to gain or exhibited marked weight losses. All animals had succumbed by 34 days.

Counts of intestinal organisms were made using the procedures outlined by Smith and Robinson (6). As can be seen from fig. 2, a very striking drop in the number of coliform organisms was noted with both levels of streptomycin within 5 days following the initiation of therapy. The decrease in the coliform count was between 100 to 10,000 times that observed with succinylsulfathiazole.

The total number of organisms (fig. 3) was also drastically reduced whereas the sulfonamide failed to have any depressing effect. The counts for all organ-isms (coliform and total) returned to normal between the seventh and twelfth days of treatment. This increase persisted for the duration of the experiment in the case of streptomycin but with the sulfonamide a secondary decrease in coliform organisms occurred. This drop was observed at a time at which the animals were in a moribund condition and 3 of an original 10 rats had succumbed. A further depression was noted when only 2 animals survived.

The fastness of the coliform organisms was determined in vitro by means of the agar-streak method. The strains of E. coli isolated from the feces of treated animals from 24 hours to 12 days after the initiation of antibiotic administration, were resistant to at least 50,000 units of streptomycin per ml. or greater. This represents at least a 1000 fold increase in resistance over that of the E. coli occurring in the controls or in the experimental animals on the day prior to the starting of streptomycin therapy; the resistance of the isolated E. coli being 7.5 to 62 units per ml.

The vitamin synthesis of strains of E. coli sensitive and resistant to strepto-

mycin was determined microbiologically. The biotin content of the supernatant fluid of the sensitive strains was 5 times that of the resistant cultures; for the cells the increase was 3 fold. On the other hand the folic acid content of the 2 cultures was about the same.

DISCUSSION. The results obtained indicate that the feeding of streptomycin in conjunction with a purified ration has a slight retarding influence upon growth; an effect that is counteracted by dried whole liver. Delayed manifestations of toxicity such as hyperexcitability and incoordination appeared to be independent of dietary treatment. Alopecia was noted in the treated animals,
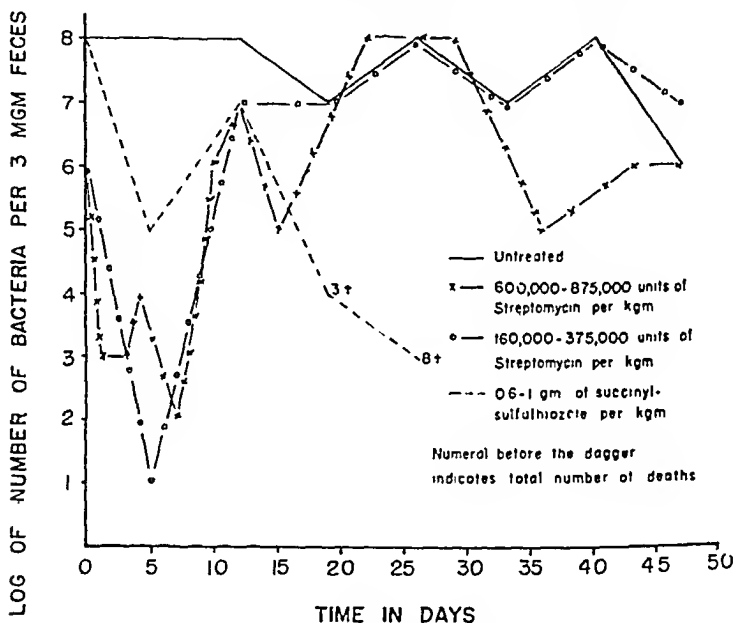
FIG. 2. EFFECT OF STREPTOMYCIN AND SUCCINYLSULFATHIAZOLE ON THE COLIFORM COUNT OF RATS

particularly in those receiving the higher level of the antibiotic; however, rats maintained on the liver containing ration with the low level of streptomycin showed the same tendency but to a much lesser extent. Experiments in progress will reveal the role of biotin as a possible protective agent. The denudation points to a possible biotin deficiency; an observation borne out by the low biotin synthesis of the resistant organisms and by the response elicited from the parenteral curative administration of biotin.

The prothrombin time was the same for both the experimental and the control groups indicating that the streptomycin treated animals did not exhibit a deficiency in vitamin K. The antibiotic likewise did not produce the leucopenia,

granulocytopenia and anemia observed in folic acid avitaminosis induced by the feeding of sulfonamide containing purified rations.

Rats receiving succinylsulfathiazole succumbed after making minimal weight gains. It would thus appear that the deficiency syndrome induced by the administration of streptomycin was not the same as that produced by succinylsulfathiazole.

Fecal counts (total and coliform organisms) were made on all treated groups. The rats receiving streptomycin showed an initial decrease in coliform bacteria that far exceeded that observed with succinylsulfathiazole. The total flora
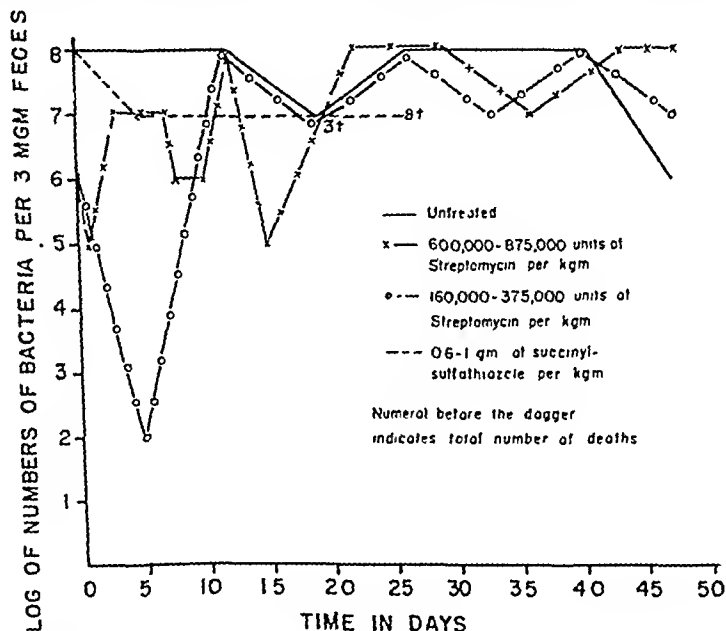
Fig. 3 Effect of Streptomycin and Succinylsulfathiazole on the Intestinal Flora of Rats

unlike the effects seen with the sulfonamide, was likewise reduced. The findings with succinylsulfathiazole are in agreement with those of Gant and co-workers (3) and of Miller (5).

The coliform counts of all streptomycin treated animals returned to normal within a few days indicating a development of fastness to the drug. This was shown to be the case by a determination of resistance *in vitro*. The new strains of *E. coli* were at least 1000 times less sensitive to streptomycin than those isolated from the feces of the animals prior to treatment or from the controls. The fastness to the antibiotic appeared within the first 24 hours of therapy although the period required for manifestation in the form of an increased count was 7-12 days.

A number of bacterial strains of the colon typhoid group have been shown readily to develop resistance *in vitro* to streptomycin and streptothricin (10).

It is rather difficult to explain the production of nutritional deficiencies (other than biotin) with the sulfonamides by the reduction in coliform bacteria. A terminal decrease was noted; however Gant et al. (3), who employed a smaller percentage of the drug in the diet reported the production of a deficiency state at a time when the count was normal. This was likewise the case in the experiments reported. It would appear that biotin is fabricated by the coliform organisms as a deficiency in this vitamin was induced by the oral administration of streptomycin.

SUMMARY

Rats receiving 160,000–375,000 units per kg. of streptomycin in conjunction with a purified diet failed to develop significant nutritional deficiencies although some evidence of toxicity was observed. Rats fed a higher level of the drug, namely 580,000–875,000 units per kg. showed signs similar to those observed in experimental biotin deficiency. Furthermore, the animals responded to biotin therapy. Microbiological analyses indicated a decreased biotin synthesis. Deficiency signs ascribable to a lack of folic acid or vitamin K were not observed.

The change in coliform count, a reduction following a return to normal, was similar to that seen with the sulfonamides; however the degree of depression was much greater. The total flora was likewise depressed with streptomycin which was not the case with succinylsulfathiazole. These findings would seem to indicate that for the temporary sterilization of the gastro-intestinal tract streptomycin might be more effective than succinylsulfathiazole.

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THE CHOLERETIC ACTION OF DEHYDROCHOLIC ACID (DECHOLIN) AND DEOXYCHOLIC ACID (DEGALOL) IN CHRONIC BILIARY FISTULA DOGS

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Dehydrocholic acid and deoxycholic acid have been isolated and made available for many years. Their properties and actions have been extensively reported upon and reviewed. Dehydrocholic acid, or sodium dehydrocholate, has been described as the least toxic and most choleretic of the bile acids and salts (1). Deoxycholic acid has been described as the most effective bile acid in aiding absorption of fats and fat-soluble vitamins from the gastro-intestinal tract (2). Based upon the estimation of a few properties of hepatic bile, dehydrocholic acid has been classified as a "hydrocholeretic" drug, since it has been reported to augment the volume but not the daily output of solids in bile (3). Such has not been our experience in a study of a wide variety of properties of hepatic bile; we have found both dehydrocholic and deoxycholic acids to augment the output of hepatic bile of normal composition in chronic, unanesthetized, biliary fistula dogs.

Sixteen dogs, of 10 to 20 kilos body weight, were prepared for the continuous collection of hepatic bile by the technique of Rous and McMaster (4). The animals were given a liberal diet of dog chow, supplemented with table scraps, and exercised daily outdoors. They soon became accustomed to the cage and dressings attached to the abdomen and quietly allowed the daily removal of bile from the condom collecting bag. In this manner, dietary and emotional factors seemed to have been satisfactorily controlled. Dehydrocholic acid and deoxycholic acid were given in the form of Decholin and Degalol respectively, both drugs being kindly provided us by Riedel-de Haen, Inc. The drugs were administered in a dose of 0.1 gm. per kilo body weight impregnated in small pieces of meat which the dogs readily swallowed. Hepatic bile was drained off, measured and aliquots submitted to analyses at intervals of 24 hours following administration of each bile acid for periods up to 10 days. Values for the first three days after administration have been averaged and summarized in table 1, since in most dogs the output of bile, increased by the administration of the bile acids, had returned to, or was returning to, normal by the end of the third day.

Upon each 24 hour sample of bile, the various estimations listed in table 1 were made. The relative viscosity was determined with the Ostwald viscosity pipette, sodium after the method of Hoffman and Osgood (5), potassium after the method of Hoffman (6), chloride after the technique of Van Slyke (7), inorganic phosphate after the method of Briggs (8), total fatty acids and the

three cholesterol fractions after the micromethod of Boyd (9), bilirubin after the technique of Thannhauser and Anderson (10) and bile acids, or salts, by an adaptation of the Mylius-Pettenkoffer reaction. All colorimetric tests were adjusted to use with the photelometer and checked by measuring percentage recoveries of known added amounts of the substance in question, in the usual manner.

Although a considerable body of work was involved and a large number of results obtained, the data may be described very briefly. The data from all experiments were tabulated, averaged and the mean changes recorded in table 1.

TABLE 1

The effect of Decholin ("N") and Degalol ("L") upon the output and properties of hepatic bile from chronic biliary fistula dogs

PROPERTY*	NORMAL	EFFECT OF DECHOLIN (' N ') AND DEGALOL ('L')					
		Day 1		Day 2		Day 3	
		N	L	N	L	N	L
24 hr volume	6 4	15 8	9 5	12 9	10 3	9 9	10 8
Specific gravity	1 0075	1 0064	1 0086	1 0064	1 0074	1 0063	1 0058
Relative viscosity	1 241	1 202	1 241	1 246	1 206	1 225	1 268
Total solids	3 78	3 72	3 69	3 64	3 75	3 66	3 69
Sodium	321	316	316	322	314	318	314
Potassium	44	43	51	42	54	42	44
Chlorides	413·	422	403	428	403	422	402
Inorganic phosphate	14	12	16	13	15	15	14
Total fatty acids	210	182	198			208	212
Total cholesterol	38	38	35			38	39
Ester cholesterol	24	20	25			24	26
Free cholesterol	14	18	10			14	13
Bilirubin	26	21	24	26	25	32	29
Bile acids	2 2	1 9	2 1	1 9	1 7	2 0	1 0

* The units were 24 hour volume ml per kilo body weight per 24 hours, total solids and bile acids gm per 100 ml of bile, viscosity was related to distilled water equals 1 00 and all others were in terms of mgm per 100 ml of hepatic bile

In all experiments, there was an increased output in the volume of hepatic bile following administration of either Decholin or Degalol. On the other hand, there was little or no change in the properties of this bile, as may be seen from the data collected in table 1. It may be concluded that while Decholin and Degalol both increase the volume output of hepatic bile in unanesthetized, chronic biliary fistula dogs, no change occurs in the concentration of any of the properties studied.

SUMMARY

Decholin and Degalol were given per ora in a dose of 0.1 gm. per kilo body weight to 16 unanesthetized, chronic biliary fistula dogs. Over a period of

three days, both drugs produced an increased volume output of hepatic bile but no significant change in its specific gravity, relative viscosity, total solids, sodium, potassium, chlorides, inorganic phosphate, total fatty acids, total cholesterol, ester cholesterol, free cholesterol, bilirubin or bile acid contents.

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A NOTE ON THE BELL AND KRANTZ MODIFICATION OF THE KNUDSON-DRESBACH CHEMICAL ASSAY OF DIGITALIS

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A recent paper by Bell and Krantz (1) has suggested that the potency of digitalis preparations may be determined chemically. They employed the Knudson-Dresbach (2) method with two modifications: A tincture prepared from the U.S.P. Digitalis Reference Standard and a 50 % dilution of this tincture

TABLE 1

Comparison of chemical and pharmacopoeial assays on three digitalis tinctures

SAMPLE NO.	PER CENT POTENCY IN TERMS OF STANDARD			
	U.S.P. XII	Chemical		Chemical as % of U.S.P. XII
		Full strength	Half strength*	
2	96	156	174	175
	84	159	175	
5	97	159	153	156
	105	158	152	
10	107	201	190	174
	126	204	190	

* The potencies obtained on 50% dilutions of the tinctures were multiplied by 2 to give the figures in this column

served as the standards, and the intensity of the color was measured with an electrophotometer. These refinements have undoubtedly increased the precision and reproducibility of the original method, but lack of specificity would seem to constitute as serious a handicap as ever in its application to any solutions other than those containing pure glycosides.

In the 18 preparations of digitalis examined by Bell and Krantz the chemical and U.S.P. XII assay agreed within the limits of experimental error frequently admitted to exist. The method is now being subjected to collaborative study by the U.S.P. Committee of Revision.

In the course of our regulatory activities we have occasion to assay numerous samples of digitalis and its preparations by the U.S.P. XII method. We have now examined 24 of these samples (16 tinctures, 8 tablets and capsules) by both the U.S.P. XII method and the colorimetric method described by Bell and Krantz. In 21 of these samples there was fair agreement between the two methods although the values for the colorimetric assay generally ran higher

than those of the U.S.P.XII assay. In the remaining 3 samples, all of which were tinctures, the potency as determined by the colorimetric assay was over 50% greater than that found by the pharmacopoeial assay. Duplicate assays were run on these three samples by both methods. Because of the difficulty in making accurate scale readings of the intense color which these tinctures developed and in the absence of data on the relation between concentration and log-extinction in this region, additional colorimetric assays were done on each of the three tinctures following dilution to half strength with digitalis menstruum. The assays on these half strength tinctures did not alter our general conclusions but did furnish the information that tinctures differ from one another in the manner in which dilution affects their chromogenicity; cf. Tinctures 2 and 10, table 1. Inasmuch as the method as outlined by Bell and Krantz makes no provision for diluting strong tinctures we have used the data obtained on the undiluted tinctures in computing the percentages in the last column of table 1.

DISCUSSION. From the present series it is impossible to tell whether the fact that these unsatisfactory results occurred only among the tinctures is anything more than coincidence. Our information on date of manufacture is incomplete but fails to indicate any relationship between the age of the respective tinctures and the magnitude of the discrepancy between the two methods. The series of 18 samples reported by Bell and Krantz included only three tinctures.

SUMMARY AND CONCLUSION

The Bell-Krantz modification of the Knudson-Dresbach colorimetric assay for digitalis gave satisfactory agreement with the U.S.P. XII assay in 8 samples of commercial tablets and capsules, but gave results from 56 to 75% higher than the U.S.P. method in three out of 16 commercial tinctures. The proposed chemical method cannot be relied on to yield results consistent with those obtained by the U.S.P. XII method for determining the potency of digitalis preparations.

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THE RESPONSE OF THE HAMSTER TO DRUGS

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Since the publication of Hsieh (1), the hamster has become an increasingly important laboratory animal for the experimental transmission of infectious diseases (2). It arises from one of the two genera—*Cricetus* and *Cricetulus*. The Chinese hamster, *Cricetulus griseus*, is easily trapped in the field, or raised in the laboratory on special diets (3). Adults usually weigh between 20 and 30 g. It is susceptible to mycobacterium tuberculosis (4, 5), pneumococcus type III (6), corynebacterium diphtheriae and its toxin (7, 8, 9), streptococcus hemolyticus (10), rabies virus (11, 12), influenza virus (13), and kala-azar (14, 15, 16, 17, 18, 19).

The Syrian or golden hamster, *Cricetus auratus*, is a larger animal. It is hardy and almost free from any characteristic odor. It multiplies quickly and thrives well with synthetic rations (20). The animal is susceptible to many virus diseases: influenza (21, 22), virus pneumonia (23), various forms of encephalitis (24, 25, 26, 27), equinine encephalomyelitis (28), the murine strain of poliomyelitis (29), mare abortion (30), lymphocytic choriomeningitis (31), and Colorado tick fever (32). The Syrian hamster is also subject to leprosy (33, 34, 35, 36, 37), tuberculosis (38, 39, 40), infections of Brucella abortus (41) and Clostridium chauvei (42), and carious lesions of the molar teeth (43). Experimental leishmaniasis (44, 45, 46, 47) and leptospirosis (48, 49) can be easily produced in the same animal.

Other species of the hamster which have served as laboratory animals are *Cricetus frumentarius* (50, 51, 52), *Cricetus cricetus* (53, 54, 55, 56), *Cricetulus furunculus* (57), *Cricetulus migratorius* (58), and *Cricetulus triton nestor* (59, 60).

The next important step is the investigation of therapeutic agents, particularly chemotherapeutics and antibiotics, in the hamster infected with different organisms and viruses. A good beginning has been made with experimental leishmaniasis (61, 62, 63, 64, 65), scabies (66), relapsing fever (67), and leptospirosis (68).

It is also necessary to study the hamster's responsiveness to drugs Reports are already available to show the indispensability of vitamins B_1, D, E, riboflavin, pyridoxine, pantothenic acid, *p*-aminobenzoic acid, inositol, biotin, choline, and possibly folic acid in the Syrian hamster (20, 69, 70, 71). Vitamin C and nicotinic acid do not appear to be required by the animal (20). The significance of hormones has been dealt with in other articles (72, 73, 74). Seasonal variations of acetylcholine action on the hamster's muscles have been demonstrated (75). Curiously, in the hamster, morphine produces a high degree of analgesia but no narcosis or respiratory depression (76). Administration of quinacrine hydrochloride is followed by the development of myo-

cardial lesions in the hamster but not in the rat, guinea pig, rabbit, dog, and monkey (77).

The purpose of the present investigation was to study the behavior of the Syrian hamster towards other well-known drugs which had not been tested. The information in this report should facilitate further pharmacologic work in this animal.

PROCEDURES AND RESULTS. Our hamsters were purchased from a dealer, but acclimated for at least a week in our air-conditioned quarters, the temperature of which fluctuated between 26.7 and 28.9°C. All experiments were performed during the months of June and July. The body weights varied from 65

FIG 1 THE HAMSTER HOLDER FOR INTRAVENOUS INJECTION
Arrows in the insert indicating the course of the superficial vein of the right hind leg

to 132 g., average 95.3 ± 1.1 g. There were more males than females—in the ratio of about 4 to 1. Their ages varied from 10 to 14 weeks. The animals were fed on a commercial diet known as "Purina Laboratory Chow" with a liberal addition of kale.

To avoid being bitten during handling, the operator should firmly seize the animal by the back of the neck. A superficial vein on the hind leg—the right leg being better than the left for a righthanded person—may be exposed by dehairing, and used for intravenous injection when the hamster is placed in a telescope-like holder, as illustrated in fig. 1. Drugs may also be very easily administered by other routes intramuscular, subcutaneous, intraperitoneal, or oral.

The Syrian hamster was found to have a mean rectal temperature of 38.7 ± 0.3°C. (15 animals), measured with a thermocouple. The mean respiratory and heart rates of normal animals were recorded as being 82 ± 5 and 286 ± 12, per minute, respectively (45 readings from 15 animals). The mean normal blood sugar in 15 hamsters was determined to be 92.5 ± 4.1 mg. per 100 cc. according to the Hagedorn-Jensen method (78).

1. Central depressants and stimulants. Three derivatives of barbituric acids were studied in the hamster starved for 16 hours. 'Sodium Amytal' (Sodium Iso-amyl Ethyl Barbiturate, Lilly) was injected both intravenously and intraperitoneally. The median anesthetic doses, or AD_{50}'s as shown in table 1, were determined to be 49.2 ± 2.4 and 61 ± 2.2 mg. per kg., respectively. By the intraperitoneal injection of a dose of 62 mg. per kg., the onset of action was 5 minutes, the duration of anesthesia averaged 25 minutes, and the recovery required 95 minutes. The AD_{50}'s of 'Seconal Sodium' (Sodium Propyl-methyl-

TABLE 1

Determination of median effective and lethal doses of seven drugs in the hamster

DRUG	ADMINISTRATION	NUMBER OF HAMSTERS USED	DOSE DETERMINED (SEE TEXT FOR EXPLANATION)
			mg. per kg.
'Sodium Amytal'	Intravenous	25	AD_{50} 49.2 ±2.4
'Sodium Amytal'	Intraperitoneal	25	AD_{50} 61.0 ±2.2
'Seconal Sodium'	Intraperitoneal	25	AD_{50} 39.0 ±1.6
Pentobarbital Sodium	Intraperitoneal	25	AD_{50} 53.0 ±2.2
Insulin	Intravenous	28	CD_{50} 61.6 ±7.4[*]
Ouabain	Subcutaneous	29	LD_{50} 23.5 ±5.5
Sodium Cyanide	Subcutaneous	29	LD_{50} 7.4 ±0.3
Strychnine Sulfate	Intravenous	20	CD_{50} 0.149 ±0.013

[*] Units per kg

carbinyl Allyl Barbituric Acid, Lilly) and pentobarbital sodium by intraperitoneal injection were found to be 39 ± 1.6 and 53 ± 2.2 mg. per kg., respectively. The average duration of action, including recovery, of the former was longer than that of the latter—210 minutes for a dose of 40 mg. per kg. as against 105 minutes for a dose of 56 mg. per kg. In order to anesthetize all hamsters (100 per cent), it was necessary to administer intraperitoneally doses of 'Sodium Amytal,' 'Seconal Sodium,' and pentobarbital sodium to the extent of 70, 45, and 62 mg. per kg., respectively. An amount of 10 per cent of each dose may be repeated from time to time for operative procedures, depending on pain reflexes and muscular rigidity.

Strychnine sulfate in suitable doses caused typical clonic convulsions by intravenous injection. The median convulsive dose, or CD_{50} (see table 1), was observed to be 0.149 ± 0.013 mg. per kg.

2. Drugs acting on the cardiovascular system. By subcutaneous injection the median lethal dose of ouabain was estimated to be 23.5 ± 5.5 mg. per kg. (see table 1). None of the injected hamsters vomited, and those that died showed tremors prior to death.

The blood pressure of 2 hamsters anesthetized with 'Sodium Amytal' was recorded with the Harvard membrane manometer. Intravenous injection of epinephrine HCl or acetylcholine chloride caused the usual rise or fall of blood pressure with tachycardia or bradycardia, respectively, as shown in fig. 2 The animal is resistant to histamine. For example, a dose of 15 μg. in the form of the acid phosphate produced a very slight fall of blood pressure.

Similar results were obtained in experiments for anaphylactic reaction. Five groups of 5 hamsters each were injected intraperitoneally with the sensitizing doses of 0 05, 0.1, 0 25, 0.5, and 1 cc of horse serum on each of 3 alternate days. At the end of 3 weeks, counting from the last dose, a final injection of 1 cc. of the same material, intraperitoneally or intravenously, resulted in scratching of the nose, ruffling of the hair, screaming, and athetoid movements, but no violent seizures similar to those occurring in the guinea pig under the same treatment All the hamsters recovered.

**EPINEPHRINE
HYDROCHLORIDE 2 μg.**   **ACETYLCHOLINE
CHLORIDE 3 μG.**
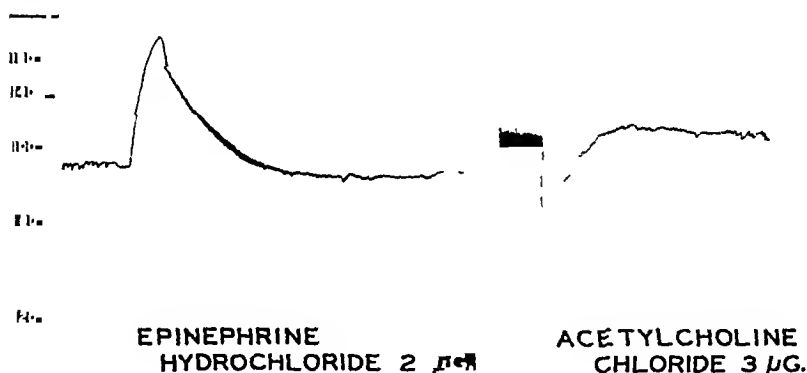
Fig 2 The Action of Drugs on the Hamster's Blood Pressure

3 Drugs acting on the isolated smooth muscle organs. Three strips of small intestines from 2 hamsters were suspended in Ringer's solution, and treated with several autonomic drugs, one after another. It was found that epinephrine HCl in concentrations of 1:2,000,000 to 1:100,000 and atropine sulfate in concentrations of 1:5,000,000 to 1:1,000,000 both relaxed the intestines, while acetylcholine chloride, 1:1,000,000, physostigmine sulfate, 1:76,923, and pilocarpine HCl, 1:6,250, all stimulated them. The effect of the last 3 could be easily antagonized by atropine sulfate Barium chloride, which has a direct action on smooth muscle fibers, caused contractions in the strength of 1:10,000

The isolated uterus of the adult or pregnant hamster has spontaneous rhythmic contractions Eleven strips of the uterus from 9 hamsters were immersed in Ringer's solution, and studied with various substances Oxytocic drugs such as pituitary extract, U S P, and ergonovine maleate produced their usual effect, namely, stimulation, as shown in fig 3. The uterus of the immature hamster 3 to 5 weeks old was less reactive to pituitary extract and ergonovine

maleate than that of adults. Barium chloride in concentrations of 1:20,000 to 1:10,000 also contracted the isolated uterus. Histamine acid phosphate had a less certain action, causing only moderate stimulation in 1:200,000 to 1:100,000 solutions. Pilocarpine HCl, 1:6,250, physostigmine sulfate, 1:76,923, acetylcholine chloride, 1:1,000,000, and mecholyl chloride, 1:1,000,000 to 1:100,000, all produced stimulating action. Atropine sulfate, on the other hand, uniformly relaxed the uterus, particularly when applied at the peak of action of the parasympathomimetic drugs. Epinephrine HCl had also an inhibitory action although by a different mechanism.

4. Insulin. A series of 28 hamsters, divided into 6 unequal groups, were injected intravenously with various dose levels of insulin. Convulsions occurred

ERGONOVINE MALEATE
1:100,000

PITUITARY EXTRACT 0.3r
0.01 UNIT PER 100 C.C.
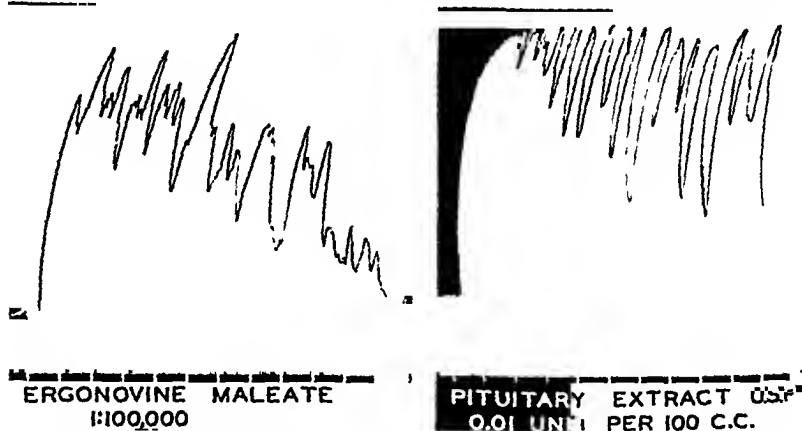
FIG. 3 THE ACTION OF DRUGS ON THE ISOLATED HAMSTER'S UTERUS

following the administration of adequate amounts. The median convulsive dose, CD_{50} (see table 1), was observed to be 61.6 ± 7.4 units per kg. The blood sugar of 10 convulsing animals, determined by the Hagedorn-Jensen method (78), varied from 19 to 47 mg. per 100 cc.—a hypoglycemia comparable to that of other mammals.

5. Sodium cyanide. A group of 29 hamsters were injected subcutaneously with various doses of sodium cyanide. The LD_{50} was computed to be 7.4 ± 0.3 mg. per kg. (see table 1).

DISCUSSION. The results of the above experiments indicate that the Syrian hamster can be effectively anesthetized with the 3 common barbituric acid derivatives, 'Sodium Amytal,' 'Seconal Sodium,' and pentobarbital sodium. The doses presented should serve as useful guides, but they may vary from laboratory to laboratory due to differences in age, environmental temperature, and other conditions (80, 81). The hamster reacts to 'Sodium Amytal' and 'Seconal Sodium' more like the mouse than the rat, dog, and man (79). Thus, 'Sodium Amytal' is shorter acting than 'Seconal Sodium' in the hamster and mouse; and vice versa, in the rat, dog, and man.

The hamster is apparently twice as sensitive to strychnine sulfate as the mouse, the data for the latter being published previously (80).

There is a natural resistance of the hamster to the digitalis-like drug ouabain. The lethal doses for the cat, rabbit, and guinea pig all amount to a small fraction of 1 mg. per kg., as shown previously (82), while the mean lethal dose for the hamster exceeds 23 mg. per kg. The susceptibility of the hamster to ouabain obviously lies between that of the mouse and that of the rat (82).

Although the hamster shows the usual response to epinephrine and acetylcholine like other mammals, it exhibits a relative resistance to histamine. This is demonstrated on both blood pressure and the isolated uterus. In the same manner the animal is not very reactive to anaphylaxis induced by horse serum.

The isolated intestine of the hamster behaves more like that of the rat and guinea pig than that of the rabbit. The peristaltic movements are small and require magnification in recording. Its response to epinephrine, acetylcholine, pilocarpine, physostigmine, atropine, and barium chloride is similar to that of the isolated rabbit's intestine.

The isolated uterus of the hamster registers spontaneous, rhythmic movements. Like that of the rat, it relaxes with epinephrine and atropine, and contracts with pituitary extract, ergonovine, acetylcholine, pilocarpine, physostigmine, barium chloride, and histamine. The immature uterus appears to be less sensitive than adult ones. Due to spontaneous contractions, even the mature uterus is not suitable for assaying oxytocic drugs.

The hamster readily develops hypoglycemic shock following intravenous injection of insulin. The blood sugar at the peak of action is comparable to that of other mammals. When judged by the median convulsive dose, the hamster is more resistant than the rabbit, mouse, rat, and dog (83).

The sensitivity of the hamster to sodium cyanide is comparable to that of the mouse and dog, but is lower than that of the rabbit (84).

The normal rectal temperature, blood sugar, respiratory rate, and heart rate have been reported in this communication. The normal blood pressure of the anesthetized hamster is in the neighborhood of 100 mm. Hg.

If the hamster is in hibernation, its response to drugs will be undoubtedly different in degree or quality, as judged from other hibernating animals, such as, the woodchuck to insulin (85). All the data of the present series of experiments were recorded at temperatures of 26.7 to 28.9°C., when the hamster was widely awake.

SUMMARY

The Syrian hamster has been studied with various drugs. The anesthetic doses of 3 well-known barbituric acid derivatives have been determined. This information will be useful for operative procedures in the laboratory.

The Syrian hamster is about twice as sensitive to strychnine as the mouse.

The blood pressure response of the Syrian hamster to acetylcholine and epinephrine is similar to that of other mammals. On the other hand, the hamster is relatively resistant to histamine and especially to anaphylactic reaction.

The isolated intestine and uterus of the Syrian hamster behave more like those of the rat towards autonomic drugs and smooth muscle stimulants.

The rectal temperature, blood sugar, blood pressure, heart rate, and respiratory rate of the normal adult Syrian hamster have been recorded.

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STUDIES ON FACTORS INFLUENCING THE TOXICITY OF NICO-TINE. THE EFFECTS OF WATER AND FOOD DEPRIVATION; DISTURBED LIVER FUNCTION AND RATE OF ADMINISTRATION

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During studies on factors which might influence the toxicity of nicotine for laboratory animals, certain ones have been encountered which seem worthy of reporting, especially because of their possible broader applicability to other toxicological investigations. These are described under several headings as follows:

I. THE EFFECT OF WATER AND FOOD DEPRIVATION ON NICOTINE TOXICITY. Crisler (1) has found that rats deprived of water for 48 hours were more susceptible to the subcutaneous administration of depressing drugs (morphine; magnesium sulfate) and somewhat less susceptible to a stimulating one (strychnine), than control animals. In similar studies on guinea pigs Carmichael (2) observed that a 48 hour period of water deprivation increased their susceptibility to subcutaneously administered tetanus toxin. In Maloney's experience (3) quick dehydration of rabbits by the intravenous administration of hypertonic glucose and sucrose solutions led to an increase in resistance to intravenously injected picrotoxin and subcutaneously injected strychnine. The general scientific import of these observations, as well as the possibility that on occasion some irregularity in watering and feeding might accidentally occur, and so influence experimental results, prompted us to study the effects of water deprivation, and in addition, starvation, on the susceptibility of mice, rabbits and rats to parenterally administered nicotine solutions.

The nicotine solutions used in these as well as in all of the experiments here reported were adjusted to pH 7.3 with HCl and concentrations so made that the dose volumes for intraperitoneal injections were: mice, 20 ml. per kg.; rats, 5 ml. per kg.; rabbits, 3 ml. per kg.; for intravenous injections: mice, 5 ml. per kg.; rabbits, 1.5 ml. per kg. Unless otherwise specified the dose of nicotine was calculated on the basis of the weight of the animal immediately prior to injection.

RESULTS (table I lists the experimental data and results): *Experiments 1 and 2 (mice)*: The mice in experiment 1 which had been deprived of water for 48 hours were significantly more tolerant to intraperitoneally administered nicotine than were the controls. Starvation for 48 hours with water allowed ad libitum (experiment 2) gave results indicating a trend towards somewhat greater tolerance on part of the starved animals, although the difference noted between the results obtained on the starved and the control mice was not statistically significant, as determined by calculating $\sigma_{D\%}$(4).

Experiment 3 (mice): The mice used in this experiment were aliquots from the same lot. Significant differences in susceptibility to nicotine given intraperi-

toneally occurred after water deprivation alone for 24 hours, and after water deprivation plus starvation for a similar period (B; E). There appeared to be no additive effect when starvation was·superimposed on water deprivation.

TABLE 1

The effect of water and food deprivation on nicotine toxicity

EXP. NO.	GROUP	NO. ANIMALS	EXP. PROCEDURE	AV. INITIAL WT.	AV. EXP. WT.	AV. CHANGE WT.	DOSE NICOTINE	MODE ADMINISTRATION	RESULTS Fatalities	Statistical significance (P = .05)
				gm.	*gm.*	*per cent*	*mg./kg.*		*per cent*	
1—Male albino mice	Control	40			25.26		8.5	I.P.	40	
	Expt'l	40	Without water 48 hr.		20.77		8.5	I.P.	7.5	S.
2—Male albino mice	Control	40		24.3	23.1	−5.0	11.0	I.P.	75	
	Expt'l	40	Without food 48 hr.	24.5	19.3	−21.3	11.0	I.P.	55	N.S.
3—Male albino mice	Control	40		20.66	20.75	+0.5	11.0	I.P.	40	
	Expt'l "A"	40	Without water 12 hr.	21.21	18.72	−11.7	11.0	I.P.	30	N.S.
	Expt'l "B"	40	Without water 24 hr.	20.82	17.42	−16.3	11.0	I.P.	10	S.
	Expt'l "C"	40	Starved 12 hr.	21.86	18.65	−14.6	11.0	I.P.	42.5	N.S.
	Expt'l "D"	40	Starved 24 hr.	21.05	17.22	−18.2	11.0	I.P.	25.0	N.S.
	Expt'l "E"	40	Without water & food 24 hr.	20.70	16.80	−18.9	11.0	I.P.	20.0	S.
	Expt'l "F"	40	"Deduction"	21.57	20.56	−4.6	11.0	I.P.	30.0	N.S.
4—Male albino mice	Control	40		27.04	27.28	+0.8	.55	I.V.	46.7	
	Expt'l	40	Without water 24 hr.	27.80	24.44	−12.1	.55	I.V.	36.7	N.S.
5—Male albino mice	Control "A"	40			16.0		13.0	I.P.	37.5	
	Control "B"	40			16.0		13.0	I.P.	30.0	N.S.
6—Rabbits, mixed sexes	Control	16		1350	1400	+3.7	15.5	I.P.	60.0	
	Expt'l	15	Without water 72 hr.	1310	1100	−16.03	15.5	I.P.	13.3	S.
7—Rabbits, mixed sexes	Control	15		1750	1530	+2.8	6.95	I.V.	26.7	
	Expt'l "A"	15	Starved 72 hr.	1520	1660	−8.5	6.95	I.V.	20.0	N.S.
	Expt'l "B"	15	Without water 72 hr.	1590	1660	−12.2	6.95	I.V.	60.0	S.
8—Male albino rats	Control	30			371.3		23.0	I.P.	70.0	
	Expt'l "A"	30	Without water 24 hr.		330.5		23.0	I.P.	60.0	N.S.
	Expt'l "B"	30	Without water 48 hr.		331.5		23.0	I.P.	73.0	N.S.

The results obtained after starvation (24 hours) alone (D), while indicating a trend toward lesser susceptibility, were not found to show a statistically significant difference from the control.

A 12 hour period of water deprivation (A) or starvation (C) did not significantly alter the toxicity of nicotine.

A lower mortality might be expected in the starved group (D) because the dose of nicotine, calculated on a weight basis, was smaller in this series in terms of living tissue than was the case with fed control mice, because of the smaller amount of material in the gastro-intestinal tract of these starved animals. To test the validity of such reasoning, the experimental group labelled "deduction" (F) was set up. This series consisted of normally fed mice, in which the dose of nicotine was determined after having deducted from the weight of each mouse the difference between the average weight of the gastro-intestinal tract of control mice and those starved for 24 hours (1.27 gm.). The figure, 1.27 gm., was arrived at by subtracting the average weight of the excised gastro-intestinal tracts of the previously referred to 24 hour starved group (D) from that of the control mice. The mortality figures for this "deduction" group, 30%, approached closely that of the 24 hour starved group (D), 25%. This indicates that the smaller amount of material in the gastro-intestinal tract of the starved mice might have been a factor in accounting for the increase in tolerance trend because of its influence on the calculated dose of nicotine. That this is at best a smaller factor in the case of the water deprived mice in which increase in tolerance to nicotine is most marked, would follow from our further observation that the gastro-intestinal tracts of these animals averaged only 0.65 gm. less than those of the controls.

Experiment 4 (mice): Contrary to the results obtained by intraperitoneal injection, deprivation of water for 24 hours did not influence the toxicity of intravenously injected nicotine for mice.

Experiment 5 (mice): Experiment 5 was done to demonstrate the extent of the variance in mortality values obtained on two groups of 40 identically treated control mice, thus enabling better appreciation of the significance of the results acquired in the various experimental groups. In one group the fatalities were 37.5%, in the other, 30%; this difference is not statistically significant. Obviously the results of this experiment and the statistical analyses used in the other experiments are mutually supportive.

Experiments 6 and 7 (rabbits): These experiments done on rabbits again show that "dehydration" by water deprivation for 72 hours increases the tolerance for intraperitoneally injected nicotine, whereas it tends significantly to *decrease* tolerance for intravenously injected nicotine. Shorter periods of water deprivation were not studied. Starvation for 72 hours was without effect on nicotine toxicity by intravenous injection.

Experiment 8 (rats): These experiments show that in the rat, water deprivation for periods up to 48 hours did not influence the toxicity of intraperitoneally administered nicotine. Why the rat should react differently from the mouse in respect to the effect of water deprivation on the toxicity of intraperitoneally administered nicotine is difficult to explain. Possibly the time interval selected may not have been sufficiently long to elicit a detectable difference.

Discussion: Crisler (1) explained the results he obtained when studying the effect of water deprivation on the intensity of action of subcutaneously injected drugs on the basis that dehydration, because of its depressing action on the

organism generally, served to potentiate the effects of depressing drugs and to antagonize the effects of stimulating ones. Because we found that the toxicity of nicotine in water deprived animals was decreased when it was administered intraperitoneally, and remained unchanged (Exp. 4) or increased (Exp. 7) when it was administered by vein, we have assumed that the tolerance encountered in the intraperitoneally injected group was due to a diminished rate of nicotine absorption from the peritoneal cavity. This might be explained on the basis of
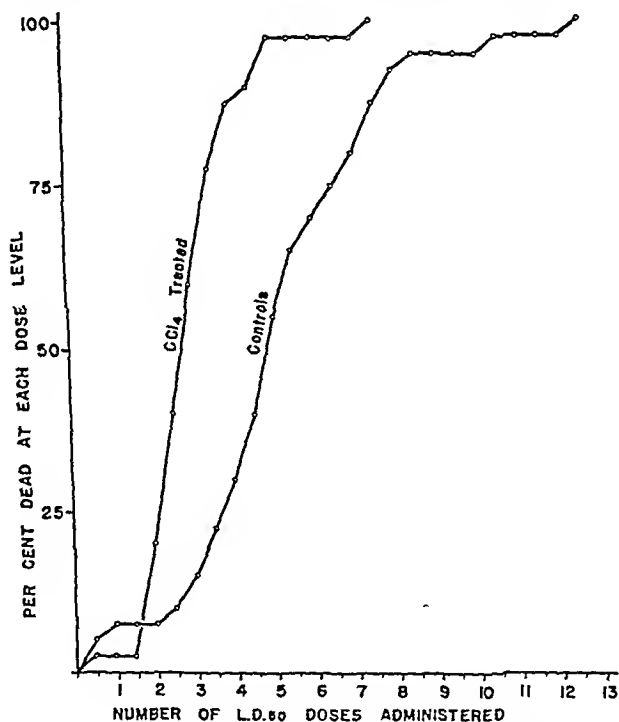
GRAPH I. THE EFFECT OF DISTURBED LIVER FUNCTION (PRODUCED BY EXPOSURE TO CARBON TETRACHLORIDE) ON THE TOXICITY OF NICOTINE FOR MICE

Dose nicotine = 5 mg. per kg. ($\frac{1}{4}$ L.D.$_{50}$), every 15 minutes, intraperitoneally.

diminished blood volume following water deprivation (5) with consequent diminution of the blood supply to the peritoneal cavity. No attempt was made to estimate the effect of water deprivation on blood volume; however, it was found that the minced carcasses of the "dehydrated" mice (Exp. 1) contained slightly less water when dried to constant weight at 105°C. than did their controls (66.64% vs. 68.73%). This, however, was not a statistically significant difference.

II. The effect of disturbed liver function on nicotine toxicity. This was studied by first inducing liver damage. Forty male albino mice were exposed to a vapor of carbon tetrachloride for a period of 60 minutes, during 40 of which the animals were completely anesthetized. Twenty-four hours later these mice, along with 40 controls, were injected intraperitoneally with 5 mg. per kg. nicotine ($\frac{1}{2}$ L.D.$_{50}$) every 15 minutes. Graph I illustrates the results obtained and shows the marked reduction in nicotine tolerance of the carbon tetrachloride treated mice as compared with the controls These findings indicating the important rôle of the liver in nicotine detoxication, are in keeping with the observations of several other groups of investigators (6, 7, 8, 9). Necropsy (gross
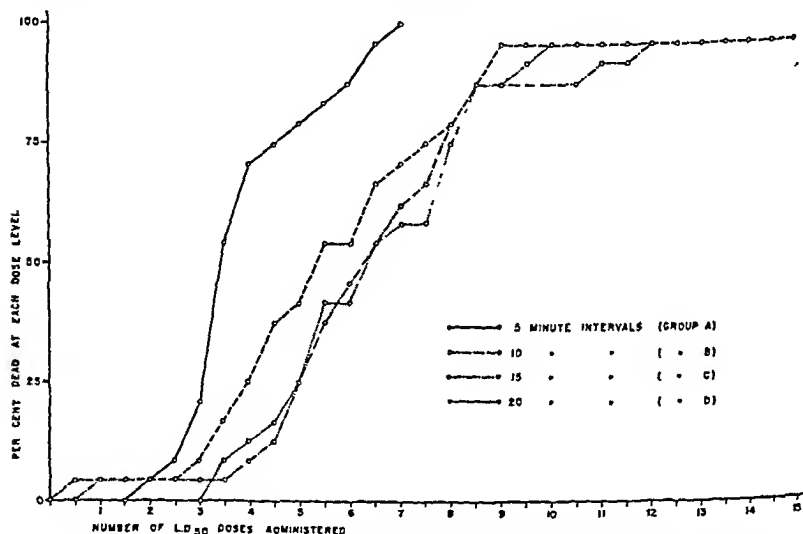
GRAPH II. The Effect of Rate of Administration on Nicotine Toxicity for Mice. Effect of Length of Interval between Doses

Dose nicotine = 5 mg per kg. ($\frac{1}{4}$ L D $_{50}$) at 5, 10, 15 and 20 minute intervals, intraperitoneally.

and microscopic) examination of the carbon tetrachloride treated animals revealed the characteristic hepatoxic action of this chemical.

The criticism could be made that the carbon tetrachloride treated animals might have been less tolerant to nicotine than the controls, because of a generalized debilitated state rather than specifically because of liver damage. To explore this possibility a series of experiments similar to the above were done, except that the mice were subjected, in the place of nicotine, to acute anoxia, a condition in which hepatic adequacy would not seem to be as much an influencing factor as the general body resistance Forty mice were treated with carbon tetrachloride as above, and these, together with control animals were exposed to a reduced atmospheric pressure (to 225 mm. Hg in 10 minutes, maintained for

7.5 minutes and to atmospheric in 5 minutes).   In this experiment there was an almost equal number of fatalities in each group: 55 per cent in the carbon tetrachloride series and 52 per cent in the control group.

III. The effect of rate of administration on nicotine toxicity   *A. Effect of length of interval between doses.*   This experiment demonstrates the rate of nicotine disposal by the mouse as related to time intervals between doses.

Ninety-six male albino mice were divided into four equal groups.   Five mg. ($\frac{1}{2}$ L.D.$_{50}$) per kg. of nicotine was injected intraperitoneally to surviving animals at intervals of five minutes (group A); 10 minutes (group B); 15 minutes (group
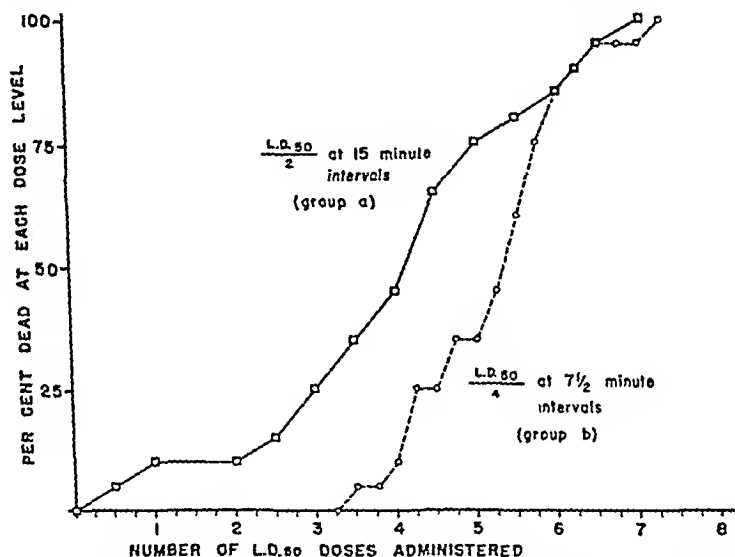
Graph III. The Effect of Rate of Administration on Nicotine Toxicity for Mice. Effect of Fractionating Dose

Dose nicotine = 5 mg. per kg. ($\frac{1}{2}$ L.D.$_{50}$) for group "a" every 15 minutes, 2$\frac{1}{2}$ mg. per kg. ($\frac{1}{4}$ L.D.$_{50}$) for group "b" every 7$\frac{1}{2}$ minutes, intraperitoneally.

C); and 20 minutes (group D).   The results are shown in graph 2.   The mean lethal dose for the entire group A was 41.0 ± 1.8 mg. nicotine per kg.; for group B, 58.3 ± 3.1 mg.; for group C, 69.4 ± 3.9 mg.; and for group D, 67.3 ± 2.8 mg. Statistically the differences are significant between all groups except between C and D.

Thus it is apparent that even small changes in the rate of administration of nicotine markedly affects its toxicity.

When calculated on the basis of mean values and in terms of mg. of nicotine, the rate of disposal for mice injected at 5 minute intervals was 0.76 mg. per kg. per minute; 0.41 mg. for those injected at 10 minute intervals; 0.28 mg. for

those at 15 minutes and 0.21 mg. for those of the 20 minute interval. These results are in general in conformity with those of Weatherby (10) who showed the relationship between rate of administration and rate of disposal of nicotine in the rabbit. However, it appears from the present studies and others previously reported (11) that the rate of disposal of nicotine in the mouse is several fold that in the rabbit.

B. *Effect of fractionating dose.* Twenty male mice were injected intraperitoneally with 5 mg. per kg. nicotine ($\frac{1}{2}$ L.D.$_{50}$) every 15 minutes (group a), and another 20 with 2.5 mg. ($\frac{1}{4}$ L.D.$_{50}$) every 7.5 minutes (group b). The results are charted in graph 3. The mean lethal dose for group a was 42.2 ± 2.7 mg. nicotine per kg.; for group b, 53.0 ± 1.4 mg. Although both groups received the same amount of nicotine during each 15 minute period, the group receiving this amount in two portions (b) showed significantly the greatest mean lethal dose, the highest L.D.$_{50}$ (5.25 vs. 4 acute L.D.$_{50}$), and a mean higher rate of detoxication per minute (.281 vs. .254 mg. per kg.). This experiment again demonstrates the importance of considering dose-interval relationships in determining maximum disposal capacities in studies of this type.

SUMMARY

1. Water deprivation was found to increase the tolerance of mice and rabbits to intraperitoneally injected nicotine, but not to intravenously injected nicotine. Water deprivation in rats for similar periods did not alter their susceptibility to intraperitoneally injected nicotine.

2. Damage of the liver by exposure to carbon tetrachloride decreased the tolerance of mice to intraperitoneally administered nicotine.

3. The rate of administration of nicotine markedly affects its toxicity.

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DETERMINATION OF DDT (2,2-BIS (p-CHLOROPHENYL) 1,1,1-TRI-CHLOROETHANE) AND ITS METABOLITE IN BIOLOGICAL MATERIALS BY USE OF THE SCHECHTER-HALLER METHOD[1]

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Effective prophylactic and therapeutic procedures in the prevention and treatment of DDT poisoning are dependent on a knowledge of its site and mode of action. Such knowledge can be quantitated only when a method of sufficient sensitivity and accuracy is available to determine small quantities of the material in tissues and excreta Several chemical methods for DDT have been developed with this in view but they all lack specificity. The earliest of these was that of Smith (1) who used the determination of organically bound chlorine to trace DDT and its metabolic products, and that in which we have used the dibrom analogue of DDT, 2,2-bis (p-bromophenyl) 1,1,1-trichloroethane by analyzing tissues for bromine. It was not, however, until the method of Schechter and Haller (2) became available that the specificity requirement was met.

The fundamental principles of this method have been thoroughly described by Schechter and Haller and only a brief summary will be given here. Upon intensive nitration DDT is converted to the tetranitro derivative. This compound gives a blue color when dissolved in benzene and methanol solution of sodium methoxide. The absorption maximum of this color in the visible range is 600 mμ. The principal metabolic product of DDT in the urine as reported by White and Sweeney (3) is 2,2-bis (p-chlorophenyl) acetic acid, hereinafter referred to as DDA. Two secondary metabolic products have been detected in this laboratory in rabbit urine. All three compounds are converted by nitration to the tetranitro p,p' dichlorobenzophenone. The tetranitro ketone when dissolved in benzene and the sodium methoxide reagent produces a red color which exhibits two maxima, the primary at 540 mμ and a secondary at 420 mμ. In this laboratory we have found DDT in all tissues examined and in the feces of animals after oral administration, but never in uncontaminated catheter urine. DDA has been found in tissue and in the urine. The other two metabolites have been detected only in the urine to the extent of 25–30 per cent of the total excretory metabolic products of DDT. Since they are extracted by the same method as that used for DDA and since they give the same color in the test they may be considered the quantitative equivalent of DDA in the urine analysis. Fig. 1 illustrates the absorption in the visible spectrum of the colors obtained with DDT and DDA.

The method as described above has been applied to tissues of animals which have received only DDT purified by repeated recrystallization from 95 per cent ethanol, melting at 108–109°C. Feeding commercial DDT which contains considerable amounts of the o,p' isomer would introduce an interfering substance since it has already been determined by Schechter and Haller that the o,p' isomer develops a color different from the p,p' isomer. The administration of other
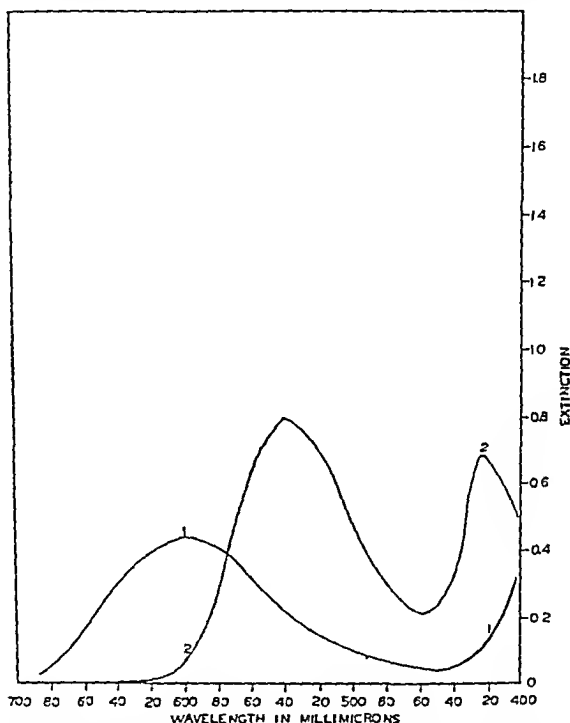
Fig. 1. Absorption Curves of DDT and DDA Colors over the Visible Spectrum
1, Tetranitro DDT 10 gamma per ml   2, Tetranitro p,p'-dichlorobenzophenone 10 gamma per ml.

halogenated compounds structurally related to DDT should also be avoided, as many of these produce interfering colors.

Several methods are available for the preparation of DDA (3, 4, 5). In this work the DDA was prepared by long hydrolysis of DDT in alkaline propylene glycol. Repeated recrystallizations from ethanol of the alkali soluble portion of the product yields DDA melting from 167–168°C. The methods for the determination of DDT and DDA in biological materials are presented in detail below.

METHODS. *Apparatus and reagents.*

1. Diethyl ether, ACS standard solvent grade.

2. Anhydrous sodium sulfate powder.

3. 0.5 N sodium hydroxide.

4. Saturated solution of sodium chloride.

5. Nitration reagent consisting of equal parts of fuming nitric acid (sp. gr.—1.5) and concentrated sulfuric acid (sp. gr.—1.8).

6. 6 N sulfuric acid.

7. Benzene, dried by redistilling and discarding the first third.

8. "Dry" methanol, prepared by drying with magnesium by the method recommended by Fieser (6).

9. Sodium methoxide solution, 10 per cent w/v in "dry" methanol. This may be prepared in either of two ways.

    a. Commercial sodium methoxide of good quality may be used. Dissolve a weighed amount in the calculated volume of "dry" methanol. (It is best to make the solution 5-6 per cent stronger than calculated since commercial sodium methoxide contains some carbonate.) Filter the solution through a coarse filter, take an aliquot, dilute it with water, and titrate with standard acid. Adjust the solution to $1.85 \pm .02$ N and store in a container protected from moisture and CO_2.

    b. If commercial sodium methoxide is not available, the reagent may be prepared by making a 4.3 per cent solution of sodium in "dry" methanol. Place the "dry" methanol in a flask fitted with a reflux condenser and keep cold by placing in an ice bath. Add the sodium portionwise through the top of the condenser. After the sodium has reacted, titrate the solution and adjust it to the required normality as described in procedure a.

10. Automatic dispensers. (Not essential but convenient.)

11. Spectrophotometer or filter photometer. The method as described has been developed with the use of a Beckman Photoelectric Quartz Spectrophotometer. However, it may be adapted to a filter photometer provided with monochromatic filters of the specified wave lengths.

12. Colorimeter cells. Use has been made of cells having a 1 cm. absorption depth and a capacity of 4 ml. For different instruments and cells the method must be adapted to fit the existing conditions.

13. Test tubes. 18 x 150 mm.

Preparation of standard curves. DDT. Prepare standard solutions of DDT in ether having concentrations of 5.0, 10.0, 20.0, 30.0 and 40.0 micrograms of DDT per ml. Pipette one ml. of these solutions into separate test tubes. Remove the ether on a steam bath using a current of air. Add two ml. of the nitration reagent to each tube and place the tubes in a steam bath for 60 minutes. At the end of this time remove the tubes and allow to cool. Dilute the mixture with 20 ml. of ice cold distilled water and transfer to a 125 ml. separatory funnel. Wash the residual contents of the tube into the funnel with another 20 ml. portion of water and then three times with 10 ml. portions of ether. Shake the funnels vigorously for about a minute to insure transfer of the nitration products into the ether layer. Remove the aqueous layer and discard. Shake the ether solution for 20 seconds with 20 ml. of chilled 0.5 N sodium hydroxide and wash twice with 20 ml. portions of saturated sodium chloride solution. Make the last separation as complete as possible without loss of the ether solution and transfer the latter through a dried cotton plug held in a small 40 mm. diameter Bunsen funnel into a dry 50 ml. Erlenmeyer flask. The cotton should be moistened with ether before filtration. Rinse the separatory funnel several times with ether and filter into the flask. Place a glass bead in the flask and remove the ether on a steam bath with a stream of air. Add to the dry residue 2.0 ml. of benzene and 4.0 ml. of the standard methoxide reagent. The blue color develops to a maximum in 10 minutes and may be read between 10 and 20 minutes after addition of the reagent using a wave length setting of 600 mμ. The zero setting of the instrument is made on a solution of 2 ml. of benzene and 4 ml. of sodium methoxide reagent.

DDA. Preparation of the standard curve for DDA is carried out in a manner identical to that described for DDT with the exception that the red color obtained is read at a wave length of 540 mμ, the point of maximum absorption.

A standard curve for each substance is prepared similar to that of fig. 2 by plotting extinction values as the abscissa and micrograms of DDT or DDA as the ordinate. These curves may be used to determine the amount of material in unknown samples directly from the colorimeter reading. The curves should be checked with fresh standards after the preparation of each new batch of sodium methoxide reagent since the color intensity varies with the concentration of this solution.
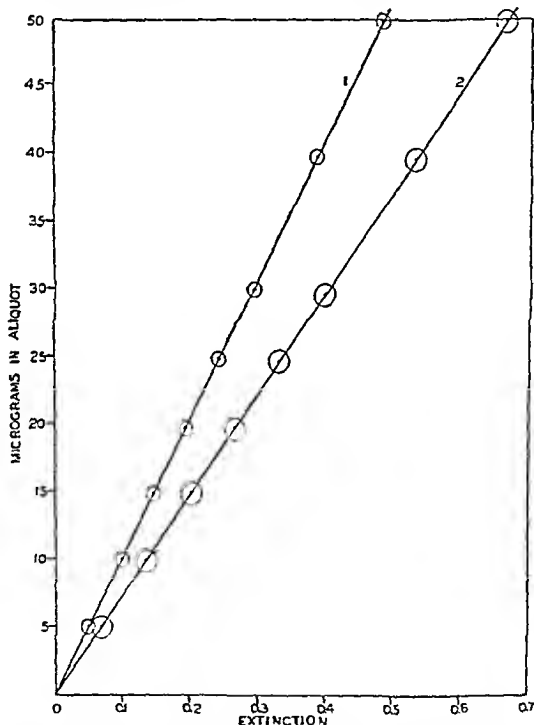
FIG. 2. RELATIONSHIP BETWEEN DDT AND DDA CONCENTRATION AND COLORIMETER READING
Curve 1, DDT color read at 600 mμ. Curve 2, DDA color read at 540 mμ.

Urine analysis. Fresh catheter urine should be used for all analyses since there is danger of contaminating the urine with DDT from feces. The volume of urine chosen depends upon the route of administration, the size of the dose, and the time interval after a single dose, and should contain approximately 30 micrograms of the acid. In this laboratory 1–10 ml. volumes of a 24-hour urine specimen are used.

Pipette the urine directly into a 125 ml. separatory funnel, acidify to pH 2 with 6 N sulfuric acid and add water to bring the volume up to 20 ml. It is usually necessary to allow the solution to stand for about 15 minutes to remove the evolved CO_2. Add an equal volume of ether and shake thoroughly to insure transfer of the DDA into the ether phase.

This may be accomplished with one extraction with ether if vigorous mixing is insured. If emulsions form they may usually be broken by the addition of a few drops of ethanol. Separate the aqueous phase as completely as possible without loss of the ether. Wash the ether with 10 ml. of distilled water followed by a washing with 10 ml. of a saturated salt solution. Transfer the ether from the top of the funnel to a test tube and remove it by evaporation on a steam bath. This may be done by portions if necessary. Rinse the funnel with several small portions of ether, add the rinsings to the test tube and evaporate to dryness using a current of air. Add two ml. of the nitration reagent to the dry residue and carry out the procedure from this point as described under Preparation of standard curves—DDA. Estimate the concentration of the DDA in the final solution directly from the standard curve and from this figure calculate the concentration in the urine sample. To convert the weight of DDA to its equivalent of DDT multiply by 1.258. Recoveries of added DDA to normal urine by this method are given in table 1.

Tissue analysis. All tissues except the adipose tissue may be analyzed by the following procedure. Since the latter contains relatively large amounts of DDT as reported by Woodard, Ofner, and Montgomery (7) (as much as 500 mg. per cent) special methods are necessary for its analysis. This will be taken up under a separate heading.

(a) *Preparing extract.* Wash the fresh tissue with a stream of distilled water from a wash bottle to remove any blood clots from the external surface, blot the surface dry and

TABLE 1

Recovery of DDA added to one ml. of rabbit urine

MICROGRAMS DDA ADDED	MICROGRAMS DDA RECOVERED	% RECOVERY
10	10.2	102
10	9.7	97
20	20.2	101
20	20.0	100
40	38.5	96
40	38.5	96

weigh immediately. Grind the tissue in a mortar with sufficient 6 N sulfuric acid to make the pH approximately 2. Add a weighed amount of anhydrous sodium sulfate equal to about twice or three times the weight of the tissue and grind to a fine powder to insure complete breaking up of the cells. Transfer quantitatively the whole sample or a weighed aliquot to a Soxhlet with a sleeve having a capacity of about 30 ml. Carry out the extraction with 40 to 50 ml. of ether for a period corresponding to six syphonings of the apparatus. Duplicate samples of each tissue should be run if two or more grams wet weight are available. For a single analysis one gram of tissue is the minimum allowable weight to be used. At the end of the period transfer the ether extract quantitatively to a 50 ml. volumetric flask and make up to the mark with ether rinsings from the extraction vessel. The extracts may be stored in the refrigerator for several days before analysis. When less than two grams of tissue are extracted in this manner concentrate the extract to a volume of 20 ml. and transfer it directly to a separatory funnel as described below.

(b) *Separation of DDT from DDA.* The above extract contains both DDT and DDA. To carry out the analysis it is necessary to separate the two. This may be done in the following way. Pipette a definite volume of the extract corresponding to approximately one gram of the original tissue into a separatory funnel and adjust the volume to 20 ml. with ether. Wash the ether twice with 0.5 N sodium hydroxide and once with distilled water using 10 ml. volumes in each case. Combine the aqueous washings in another

separatory funnel, wash the latter once with 10 ml. of ether and transfer the aqueous layer to another funnel. Combine all ether extracts. The DDT remains in the ether solution and the DDA is carried over into the alkaline extract. The analysis is carried out separately for each substance as given below.

(c) *Analysis for DDT.* Dry the ether layer containing the DDT by shaking twice with 10 ml. of saturated salt solution. Transfer the dried ether quantitatively to a test tube with several washings of the funnel with ether. Evaporate the ether and if necessary dry the residue by the addition of a small amount of acetone which is subsequently removed by boiling and using a current of air. Place the test tube containing the dried residue in an ice bath, add approximately two ml. of the nitration reagent for every gram of original tissue represented by the extract and allow to remain in the ice bath for 30 minutes. Remove the test tube, bring it to room temperature and then place it in a steam bath for 60 minutes. The procedure from this point is identical to that described under Preparation of standard curves—DDT. The amount of DDT present may be read directly from the standard curve after the subtraction of the blank if necessary. The discussion of the determination of the blank will be presented under a separate heading.

(d) *Analysis for DDA.* Adjust the combined alkaline washings from above to a pH of 2 with 6 N sulfuric acid. Add an equal volume of ether and extract the solution by vigorous shaking. Separate the aqueous layer and discard it. Wash the ether layer once

TABLE 2

Recovery of added DDT and DDA to one gram of rabbit liver

LIVER NO.	MICROGRAMS DDT ADDED	MICROGRAMS DDT RECOVERED	% RECOVERY DDT	MICROGRAMS DDA ADDED	MICROGRAMS DDA RECOVERED	% RECOVERY DDA
1	5.0	5.0	100	2 0	2.1	105
2	5.0	5.2	104	4 0	4.2	105
3	10.0	9.7	97	2.0	2.0	100
4	10.0	10.0	100	6 0	5.7	95
5	15 0	15 0	100	4.0	4.2	105
6	15 0	14.7	98	6.0	6.0	100

with distilled water and twice with saturated salt solution using 10 ml. volumes for each washing. Transfer the ether quantitatively to a test tube, evaporate the ether, and dry the residue with acetone if necessary. Chill the tube and add the nitration reagent in the proportions described for DDT. The remainder of the procedure is identical to that described under Preparation of standard curves—DDA.

Recoveries of both DDT and DDA when added to the same tissue and separated in this manner are given in table 2.

(e) *Estimation of the blank.* It has been found that when less than 1 mg. per cent or 10 micrograms per gram of either DDT or DDA are present in tissues or urine the recoveries are consistently 10 to 20 per cent high. This may be directly attributable to the yellow color produced on nitration of extracts of normal tissue or urine. Indeed the observed variations in recoveries at higher concentration may be due in part to differences in the blank from one animal to the next. No means has yet been found for successfully eliminating this undesirable feature of the method. We have estimated the amount of the blank by reading the solution at a spectral point where the blank absorption is greater than that of DDT or DDA and relating this reading to that of the blank at the spectral points used for the DDT and DDA analyses, viz. 600 mμ and 540 mμ respectively. The spectral point which meets this requirement is 465 mμ. Normal tissues from both rats and rabbits have been carried through the method to determine the consistency of the blank. It was found that within reasonable limits the extinction value at 600 mμ was ½ of the reading at 465 mμ. Likewise the reading at 540 mμ was ½ of that at 465 mμ. Table 3 contains data illustrating

this point. In practice the following procedure is adopted. Read at 465 mμ when the extinction value at 600 mμ for the sample containing DDT is less than that representing 10 micrograms/gram of tissue. Divide the reading at 465 mμ by 6 and subtract the quotient from the reading at 600 mμ. Refer this corrected extinction value to the standard curve for estimation of the amount of DDT. If in determining DDA the same criterion is met then the reading at 465 mμ is divided by 3 and the quotient is subtracted from the reading at 540 mμ to obtain the corrected reading for DDA.

(f) *Analysis of adipose tissue.* By far the most accurate and reproducible results are obtained when analyzing fat for DDT since the tremendous concentration in the fat necessitates the analysis of very small aliquots with consequent disappearance of interfering colors. Extract one gram of the fatty tissue with ether. This may be done conveniently by grinding with a small amount of washed sand and 10–20 ml. of ether. Repeat several times and decant the ether without loss into a 50 ml. volumetric flask after each grinding. When the fat is completely extracted leaving a small deposit of connective tissue the volume is adjusted to 50 ml. Take one milliliter of this solution for the nitration as previously described. A larger or smaller aliquot may be used depending upon the expected concentration. If it is desired to analyze for DDA the remainder of the solution is transferred

TABLE 3

Extinction values at different spectral points of normal rat livers in the Schechter-Haller procedure

LIVER NO., 1 CM. ALIQUOTS	EXTINCTION					
	DDT procedure			DDA procedure		
	465 mμ	600 mμ	465/600 mμ	465 mμ	540 mμ	465/540 mμ
1	.029	.005	5.8	.038	.013	2.9
2	.033	.006	5.5	.042	.015	2.8
3	.042	.010	4.2	.032	.011	2.9
4	.031	.005	6.2	.031	.010	3.1
5	.026	.004	6.5	.027	.008	3.4
6	.034	.005	6.8	.031	.010	3.1
Average			5.8			3.0

to a separatory funnel and extracted with alkali. The alkaline solution is then analyzed for DDA by the described procedure.

DISCUSSION

The method as described is of an empirical nature. Changes may be made as it seems advisable to the individual operator but once a standard procedure has been adopted it should be followed in every detail. In this laboratory we have used the method in determining DDA in a large number of rabbit urines. Analysis of the data from duplicate determinations ranging from 1 microgram to 40 micrograms in 1 ml. of urine gives a standard deviation ±0.5 micrograms. A sufficient number of tissue analyses have not been made to give a satisfactory calculation of the precision for the DDT analysis. However, determination of standards in duplicate as well as recovery experiments from tissue with DDT indicate that the determination of DDT is considerably more precise than that of DDA. The accuracy of the methods of both DDT and DDA are indicated from the recovery data in table 2.

The possibility of the presence of metabolites other than those already mentioned cannot be overlooked. However, the method is applicable to those discussed above. There could be others which do not produce colors when subjected to this test. Investigation of this point is under way.

To guide the investigator in his analysis the following tissue levels of DDT have been found in rabbits after single oral doses in corn oil of 200 mg./kg.: blood, 0.5–5.0 mg. %; liver, 1.0–3.0 mg. %; kidney, 2.0–5.0 mg. %; brain, 1.0–3.0 mg. %; perirenal fat, 10.0–50.0 mg. %. The tissue values of DDA range close to 10 per cent of the DDT concentration in most cases. The urine concentration of DDA varies from a high of 5.0 mg. per cent down to 0.5 mg. per cent during the first few days after administration but excretion has been detected as long as 16 days after a single dose of DDT.

SUMMARY

The colorimetric method of Schechter and Haller for DDT has been adapted to the determination of DDT and its known metabolite in biological materials. The method is fast and reliable down to 1.0 mg. per cent of either DDT or DDA in tissues or urine. Lower concentrations may be determined by the subtraction of an estimated blank with but a slight decrease in accuracy. The method is empirical and requires careful standardization of technic and reagents. An accuracy of 5 per cent can be obtained with DDT and 10 per cent with DDA. The standard deviation of duplicate analysis is ±0.5 micrograms for DDA and less for DDT.

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